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Characterization of *Sphingomonas paucimobilis* ANT 17, an Oil Degrading Bacterial Isolate From Antarctica

by

Cheryl Anne Paulette Baraniecki

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

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C. Bar

Cheryl Anne Paulette Baraniecki
Apartment 301
17715 – 64 Avenue
Edmonton, Alberta
T5T 2J9

January 20, 2000

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Characterization of *Sphingomonas paucimobilis* ANT 17, an Oil Degrading Bacterial Isolate From Antarctica" submitted by Cheryl Anne Paulette Baraniecki in partial fulfillment of the requirements for the degree of Master of Science in Microbiology and Biotechnology.

J. M. Foght (supervisor)

P. M. Fedorak

M. A. Pickard

M. A. Naeth

Date: Jan 24/2000

ABSTRACT

The Antarctic bacterial isolate *Sphingomonas paucimobilis* ANT 17 has the ability to degrade crude oil in cold and growth-limiting environments. ANT 17 grows at temperatures from 1°C to 35°C. The optimum pH for growth of ANT 17 is near 6.4 at 22°C, but at colder temperatures the optimum pH is less defined. Seventy-five aromatic and non-aromatic substrates were incubated with ANT 17, and the majority served as growth or cometabolic substrates. For example, phenanthrene was mineralized to CO_2, but fluoranthene and pyrene were partially oxidized. ANT 17 grew on and degraded the aromatic fraction of several crude oils and a refined oil product under cold and nutrient-limiting conditions, but did not grow on the saturate fraction. Molecular studies revealed that ANT 17 was phenotypically stable and plasmidless, suggesting that the genes required for aromatic degradation are chromosomal. ANT 17 may be a good model for studying cold climate bioremediation, due to its broad aromatic substrate range and ability to grow at cold temperatures.

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LIST OF ABBREVIATIONS

General

CHEF – contour clamped homogeneous electric field

EPA – Environmental Protection Agency

FAME – fatty acid methyl ester

GC – gas chromatography

GC-FID – gas chromatography with flame ionization detector

kb – kilobases

kbp – kilobase pairs

LPS – lipopolysaccharide

PAH(s) – polycyclic aromatic hydrocarbon(s)

PFGE – pulsed field gel electrophoresis

TCA – tricarboxylic acid cycle

Media

ddH_2O – doubly distilled water

NAG – nutrient agar + 0.5% glucose

NBG – nutrient broth + 0.5% glucose

QTSB – quarter-strength TSB

TSB – trypticase soy broth

Reagents and chemicals

ASMB – Alberta Sweet Mixed Blend crude oil

BCA – bicinchoninic acid

BSA – bovine serum albumin

C – carbon

DCM – dichloromethane

DMF – N,N-dimethylformamide

EDTA – ethylenediamine tetraacetic acid

N, P – nitrogen and phosphate nutrient amendment

Reagents and chemicals, continued

NH_4^+, P – ammonium and phosphate nutrient amendment

NO_3^-, P – nitrate and phosphate nutrient amendment

NW – Norman Wells crude oil

P – phosphate nutrient amendment

PBCO – Prudhoe Bay crude oil

SDS – sodium dodecyl sulfate

TMPD – tetramethyl-*p*-phenylenediamine

1. INTRODUCTION

1.1. Petroleum contamination

As a consequence of utilizing petroleum and its refined products to satisfy our energy requirements, contamination of the environment has occurred and will continue to occur. The removal and transport of petroleum from reservoirs (both on land as well as from offshore drilling sites), refining crude oil to useable products and the eventual disposal of used products results in contamination of ecosystems with many different compounds. Although petroleum has always entered the environment through natural seepage from reservoirs and photosynthetic hydrocarbons produced by plants, the amount entering the environment by natural sources is much less than the amount of petroleum introduced into the environment by industrial practices or anthropogenic sources (Floodgate, 1984).

The results of this contamination are diverse with often severe consequences. Oil floating on receiving waters is destructive to birds and aquatic life. In addition to killing effects, subtle effects on marine life can be observed (Alexander, 1999). These include the disruption of feeding and mating behavior of higher organisms by masking chemoreception, as well as carcinogenic effects of compounds resistant to detoxification, such as polycyclic aromatic hydrocarbons (PAHs), which accumulate through the food chain. The world's oceans are the largest and ultimate receptor of petroleum pollution (Atlas, 1981). Floodgate (1984) reviewed the effects and fates of petroleum in marine ecosystems and found that the largest input of petroleum into the oceans is from rivers and urban runoff; other major inputs are from the shipping industry. The residence time of petroleum contaminants is greater in estuaries and shelf waters than in the open ocean as many of the contaminants will settle. Petroleum in marine ecosystems exists as slicks, emulsions, in solution, and as tar balls. Freshwater ecosystems are contaminated directly, by rainfall from atmospheric contaminants or by runoff, and these contaminants accumulate in the air:water interface of rivers and lakes as well as in sediments (Cooney, 1984). Freshwater contaminants are subject to the formation of slicks, emulsification, and solublization, much like marine contamination.

In soil ecosystems, petroleum contamination does not spread horizontally as in aquatic ecosystems; instead the contaminants spread vertically. Contamination of terrestrial sites can be classified as surface and groundwater (or sub-surface) contamination. Sources of petroleum contamination are from accidental spills on land, especially from transportation within pipelines, production, and storage, as well as from natural seeps (Bossert and Bartha, 1984). Other less obvious sources of petroleum contamination arise from oiled roads and from wood preservatives such as creosote which are made up of 85 to 90% PAHs (Cerniglia, 1992). The effects of petroleum contamination on plants include direct toxicity, oxygen depletion by hydrocarbon degrading bacteria and the resulting production of H_2S. Soil invertebrates are also adversely affected by petroleum contamination due to direct contact toxicity, blockage of organs of respiration, as well as reduced oxygen concentrations.

1.2. Composition and properties of petroleum

Petroleum is formed by the transformation of sedimented organic debris to liquid and gaseous hydrocarbons caused by heat in the sedimentary bed. Compaction forces this matter into adjacent permeable rock, which forms a reservoir for the hydrocarbons. Due to the organic origin of petroleum, it is a complex mixture of hydrocarbons. These can be fractionated into a saturated or aliphatic fraction (paraffins), aromatic fraction, asphaltenes, and a polar or resin fraction (Atlas, 1981) that is composed mostly of aliphatic, alicyclic, and aromatic hydrocarbons, as well as non-hydrocarbon components (containing sulphur, nitrogen, and oxygen) such as thiols, heterocyclic nitrogen and sulphur compounds, metalloporphyrins, carboxylic acids, and phenols (Atlas, 1981). Alkenes are found in refined petroleum but not in crude oil (Cooney, 1984). Examples of the types of compounds found within petroleum are illustrated in Figure 1.1. Crude oils contain several hundred individual components, and vary with origin. Other properties that can vary with origin include viscosity, density, and pour point.

"Weathering" is the result of chemically and biologically induced changes in the composition of petroleum (Atlas, 1981), including evaporation, photooxidation, and microbial degradation (Floodgate, 1984). As weathering occurs, the oil becomes more viscous and dense. In addition, as readily degraded compounds are removed, the residual

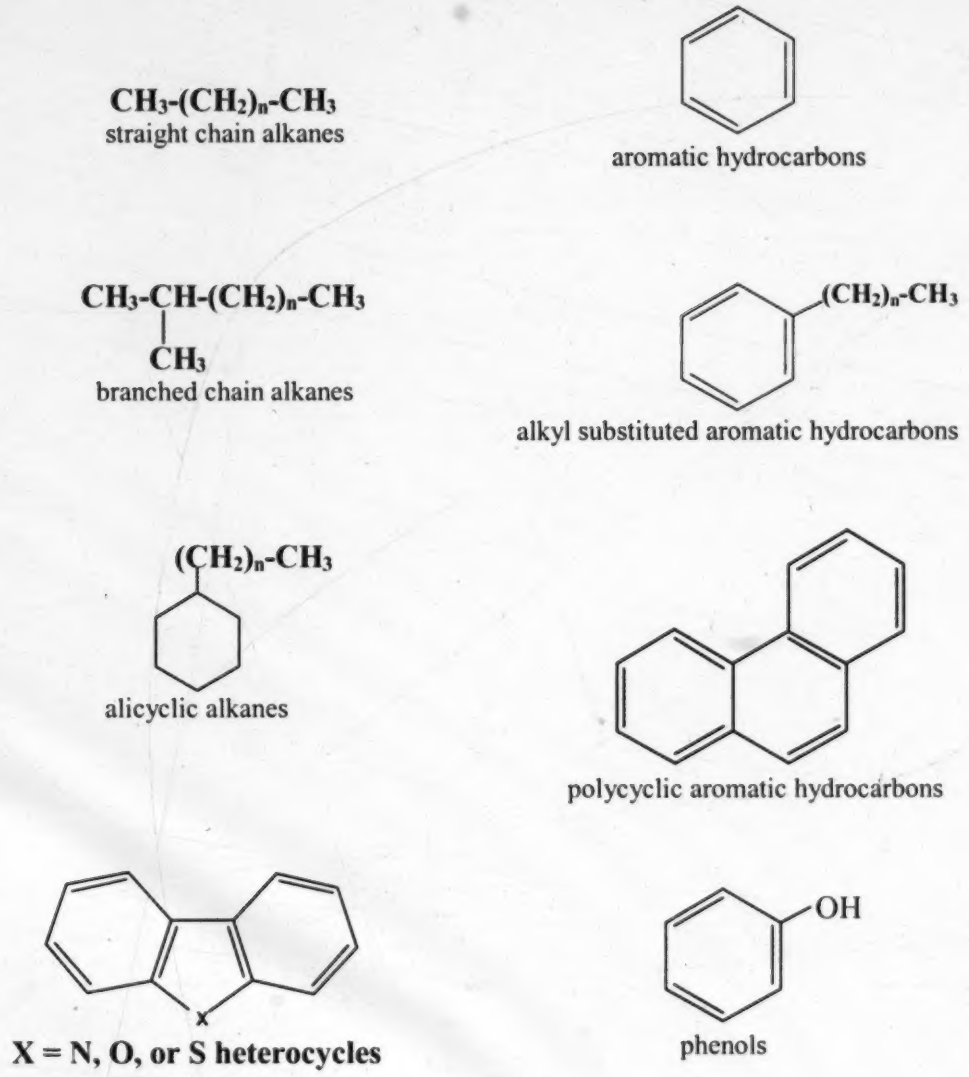

Figure 1.1. Examples of the types of compounds found within petroleum.

oil becomes more resistant to microbial attack, but also is less susceptible to migration in the environment. The remaining discussion will focus on the biological processes involved in the weathering of petroleum, biodegradation pathways utilized, and limitations to these processes.

1.3. Bioremediation of petroleum contaminated sites

Methods for remediating contaminated sites have traditionally involved physical and chemical methods. These methods involve removing contaminated soils to new sites, incineration of the contaminated soil, or chemically extracting the pollutant from soil, all of which are invasive and costly. For instance, the cost of transporting large volumes of soil to a treatment facility is greater than the cost of treatment within the contaminated site itself. In addition, these methods are not suitable for marine, surface, or groundwater contamination. Cleanup of marine oil spills has involved dispersing the oil with detergents or sinking the oil with chalk or siliconized sand which is merely cosmetic, as the contamination still exists in sediments. It is for these reasons that the interest in utilizing *in situ* remediation techniques has increased. One such *in situ* method involves the use of microorganisms (bacteria and fungi) for the purpose of remediating a contaminated site through a process known as bioremediation, which can be applied to surface soil, subsurface soils, aquifers as well as marine contamination (Atlas and Bartha, 1992).

Bioremediation involves the use of microorganisms with the goal of degrading a pollutant to either a less harmful or less toxic metabolic product (transformation) or to completely degrade the pollutant to CO_2, water, and biomass (mineralization). Bioremediation can be applied to many pollutants from industrial and commercial sources such as pesticides, waste chemicals from a variety of manufacturing industries, heavy metals and mining wastes, and petroleum products. The following discussion will focus on the bioremediation of petroleum and petroleum-related compounds.

Many bacteria and fungi are known to degrade compounds found within. This has been extensively reviewed in the literature (Cerniglia, 1992; Sutherland *et al.*, 1995; Shuttleworth and Cerniglia, 1995; Atlas and Bartha, 1992, Leahy and Colwell, 1990). These organisms have been identified from diverse environments spanning marine,

freshwater, and soil habitats. Many of the hydrocarbon-degrading isolates belong to the genera *Pseudomonas*, *Arthrobacter*, *Micrococcus*, *Flavobacterium*, *Mycobacterium*, *Sphingomonas*, *Rhodococcus*, *Nocardia*, *Vibrio*, *Beijerinckia*, *Aspergillis*, *Cunninghamella*, *Phanerochaete*, and *Penicillium*.

Interest in microbial biodegradation pathways was initiated during the 1950s and 1960s. Petroleum was investigated as a potential low-cost carbon source for the production of single cell protein as an alternative food source for livestock (Atlas and Bartha, 1992). With the increase in cost of petroleum and the realization that the removal and transport of petroleum resulted in contamination problems of the environment, interest in the metabolism of petroleum was redirected toward biodegradation of hydrocarbons for the purpose of remediation.

1.3.1. Alkane and alicyclic hydrocarbon biodegradation pathways

The biodegradation of straight chain and branched alkanes has been extensively studied and reviewed (Atlas, 1981; Singer and Finnerty, 1984b; Atlas and Bartha, 1992). A general degradation pathway for *n*-alkanes (Figure 1.2) can be described as beginning with the formation of a primary alcohol via monoterminal oxidation by a monooxygenase. Two other methods are known to convert an *n*-alkane to a primary alcohol (Figure 1.2). Dehydrogenation of *n*-alkanes can occur to form an alk-1-ene intermediate followed by rehydration to form a primary alcohol. Hydroperoxidation incorporates molecular oxygen into the *n*-alkane via a dioxygenase, which results in the formation of an *n*-alkyl hydroperoxide. This is then reduced to form a primary alcohol. Following the formation of a primary alcohol, the formation of an aldehyde (by an alcohol dehydrogenase) and a carboxylic acid (by an aldehyde dehydrogenase) occurs as shown in Figure 1.2.

An alternate to monoterminal oxidation in the formation of a carboxylic acid product is subterminal oxidation (Figure 1.3a). This scheme is more common in fungi and yeasts than in bacteria. The formation of a secondary alcohol results from the subterminal oxidation of the *n*-alkane. This is further oxidized to form a ketone and a subsequent

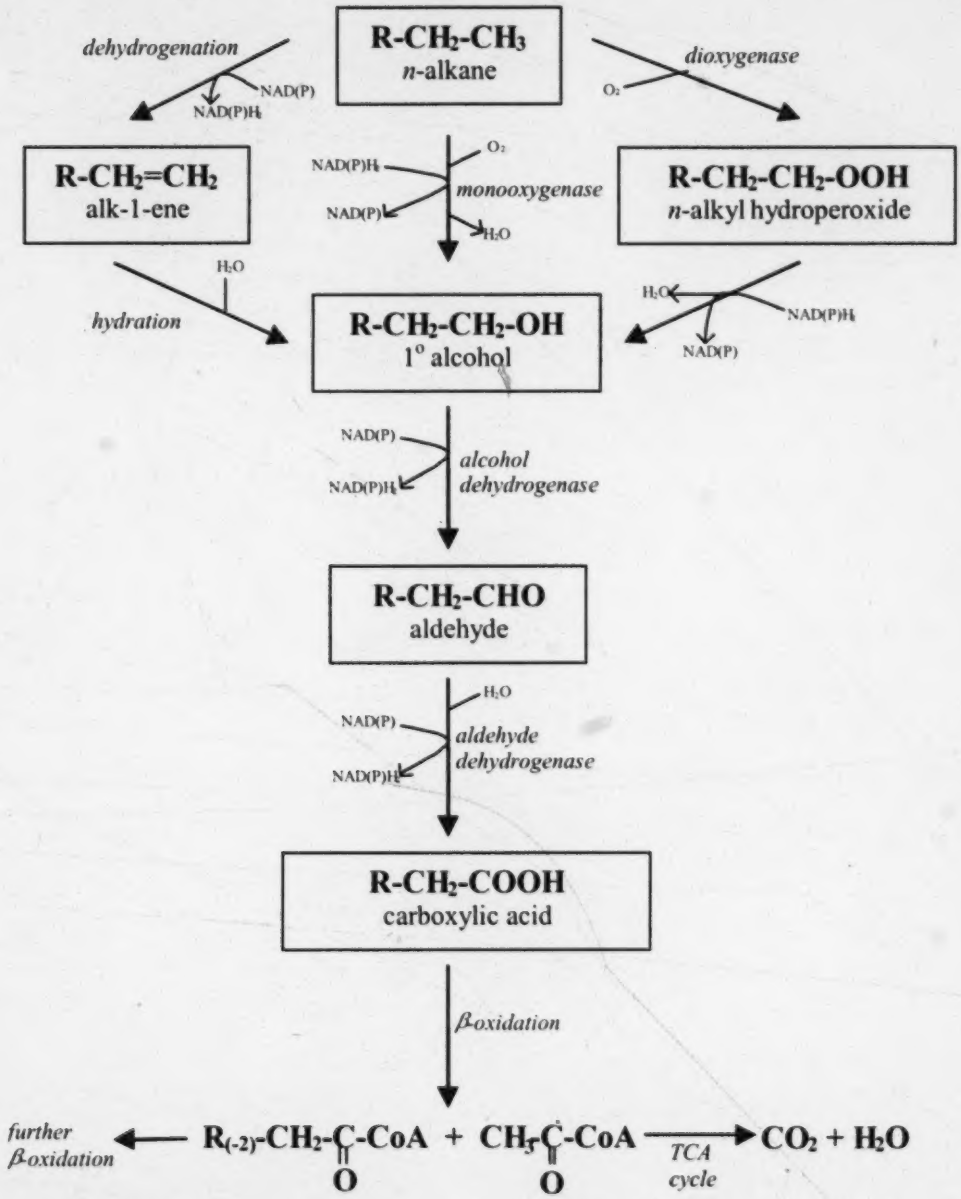

Figure 1.2. Three mechanisms of monoterminaloxidation of *n*-alkanes to a primary alcohol. Subsequent oxidations to an aldehyde and carboxylic acid as well as further degradation through β-oxidation and the TCA cycle are also shown. (Singer and Finnerty, 1984b).

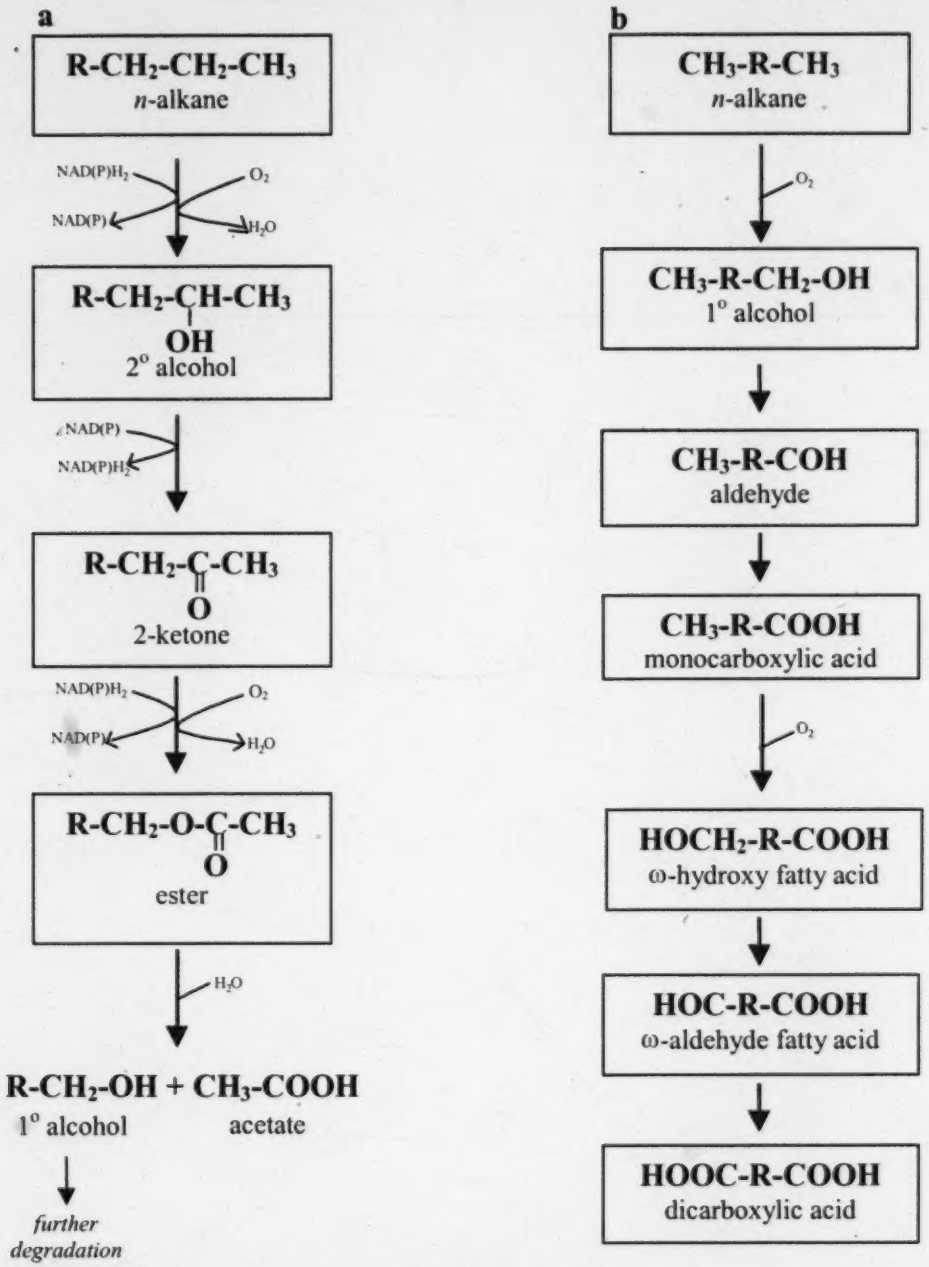

Figure 1.3. Alternatives to monoterminal oxidation of *n*-alkanes. **a.** subterminal oxidation. **b.** diterminal oxidation. (Singer and Finnerty, 1984b).

ester. The ester is hydrolyzed to a primary alcohol and acetate. If subterminal oxidation occurs at another C atom within the *n*-alkane, a carboxylic acid longer than acetate may be formed. The resulting carboxylic acid (shown as acetate in Figure 1.3a) can be used via the tricarboxylic acid (TCA) cycle, but the resulting primary alcohol must be further transformed to a carboxylic acid as described earlier. In some cases diterminal oxidation (ω-oxidation) occurs (Figure 1.3b), resulting in the formation of a dicarboxylic acid. This occurs as monoterminal oxidation proceeds on both ends of the *n*-alkane. This degradation scheme is utilized to attack branched or methyl-substituted alkanes.

All of these methods of oxidation of an *n*-alkane to a carboxylic acid result in the ability to further metabolize the carboxylic acid (Figure 1.2) through β-oxidation to form acetyl CoA and two-carbon-unit-shorter fatty acids. Acetyl CoA is converted to CO_2 through the TCA cycle. When an *n*-alkane has undergone the complete process of oxidation, dehydrogenation, and β-oxidation with the formation of CO_2 and H_2O via the TCA cycle, mineralization has occurred. When the process is halted at any of the steps prior to the formation of CO_2, transformation has occurred. Transformation of *n*-alkanes has certain biological results. Generalizations have emerged regarding the composition of cellular and extracellular fatty acids based on the carbon number of the alkane transformed (Singer and Finnerty, 1984b). Growth on odd-carbon numbered alkanes results in odd-carbon numbered cellular fatty acids and even-carbon numbered alkanes results in even-carbon numbered cellular fatty acids.

The metabolism of alicyclic hydrocarbons has been extensively reviewed (Perry, 1984; Atlas and Bartha, 1992). In general, the alicyclic hydrocarbon is oxidized to form an alicyclic alcohol via a monooxygenase (Figure 1.4). Dehydrogenation leads to the formation of a ketone. Additional oxidation often by a different monoxygenase inserts oxygen into the ring forming a lactone. The ring is then cleaved by hydration to form a ring cleavage product consisting of an alcohol and a carboxylic acid. The alcohol is further metabolized to a dicarboxylic acid, which then is able to undergo β-oxidation. Cometabolism (which can be defined as the transformation of a compound by a microorganism that is unable to utilize the substrate as source of energy; Alexander, 1999) is often required for this process. Alkyl substituted alicyclic hydrocarbons are often more readily degraded than their unsubstituted counterparts. Even-numbered *n*-

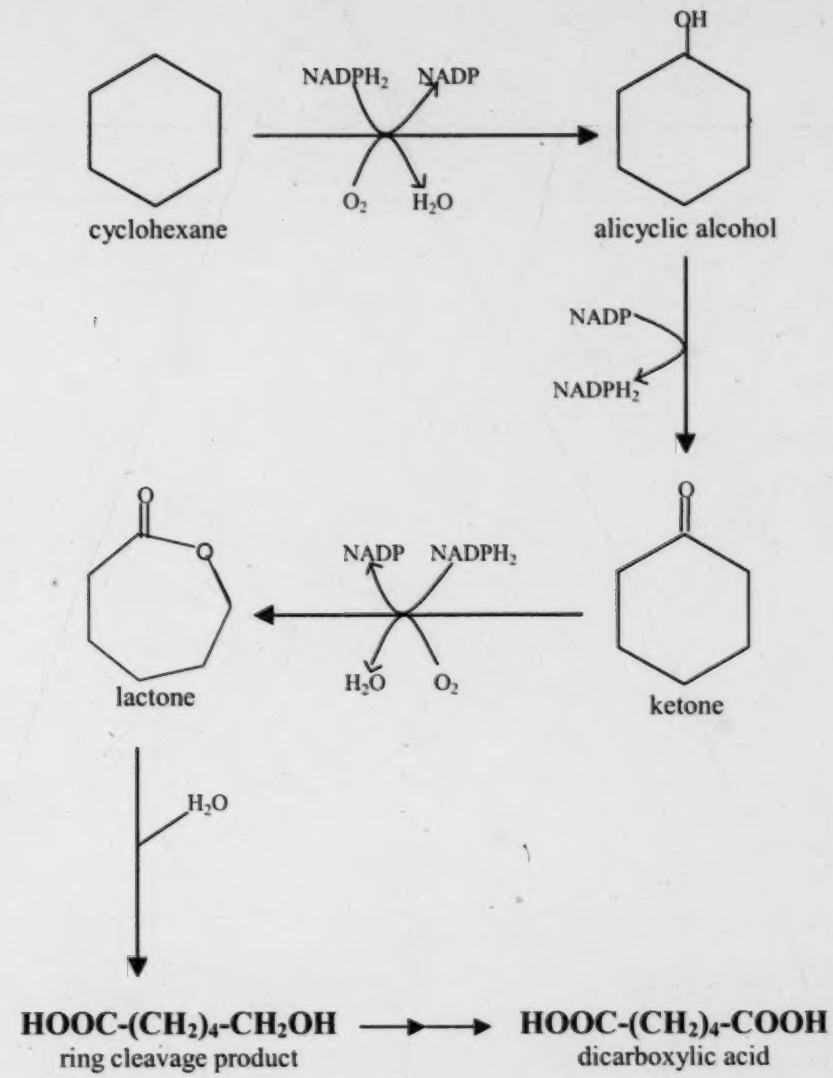

Figure 1.4. Metabolism of cyclohexane, a representative of alicyclic hydrocarbon degradation. (Perry, 1984).

alkane side chains are more resistant to ring cleavage than odd-numbered carbon *n*-alkane side chains, as the side chain is attacked through β-oxidation.

1.3.2. Aromatic hydrocarbon biodegradation

The degradation of aromatic hydrocarbons, and PAHs in particular, is of great interest. Several PAHs are toxic, mutagenic, and carcinogenic and have been designated as priority pollutants by the EPA. Figure 1.5 illustrates various PAHs commonly studied. The possible fates of PAHs in the environment include volatilization, photo- and chemical- oxidation, bioaccumulation, adsorption to soil, and microbial degradation (Cerniglia, 1992). Microbial degradation can be complete (mineralization to CO_2) or incomplete (transformation to oxidized products by either individual organisms or communities). Microbial degradation pathways for aromatic compound biodegradation have been extensively reviewed (Cerniglia, 1984; Cerniglia, 1992; Sutherland *et al.*, 1995; Atlas and Bartha, 1992) and much is known regarding the pathways utilized for toluene, naphthalene, phenanthrene, anthracene, and acenaphthene, whereas less is known about the higher molecular weight compounds. Oxygen is a general requirement to initiate enzymatic attack on PAH rings. Furthermore, metabolism of PAHs are highly regio- and stereo-specific (Cerniglia, 1992).

Initial oxidation of aromatic hydrocarbons by bacteria usually involves the action of a dioxygenase, which incorporates the two atoms of molecular oxygen to produces a *cis*-dihydrodiol (Figure 1.6). Dioxygenase enzymes are multi-component in nature consisting of a flavoprotein, an iron-sulphur protein, and a ferredoxin (Cerniglia, 1984). Once the *cis*-dihydrodiol is formed, it is further oxidized to a catechol. Oxidative cleavage results in either *ortho* cleavage of the aromatic ring between the two hydroxyl groups (to form *cis,cis*-muconic acid) or *meta* cleavage of the ring adjacent to the two hydroxyl groups (to form 2-hydroxy-*cis,cis*-muconic semialdehyde). These are subsequently degraded to intermediates of the TCA cycle as shown in Figure 1.6. Variations of this pathway can include the oxidation of alkyl substituted aromatic hydrocarbons, where the ring is oxidized adjacent to the alkyl substitution to form an alkylated catechol which is then oxidized by ring fission. Another possibility of attack on an alkylated aromatic

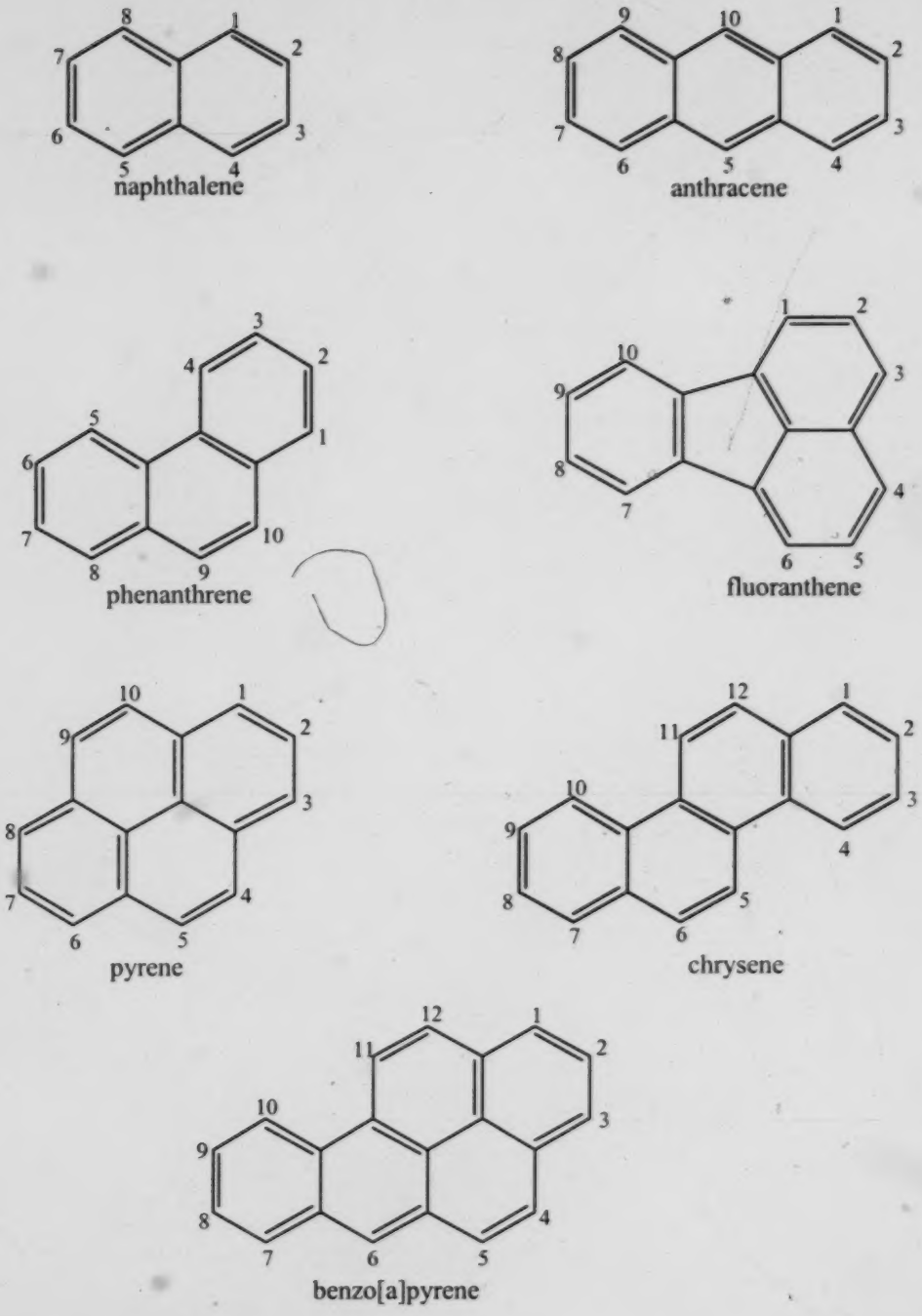

Figure 1.5. PAHs commonly studied.

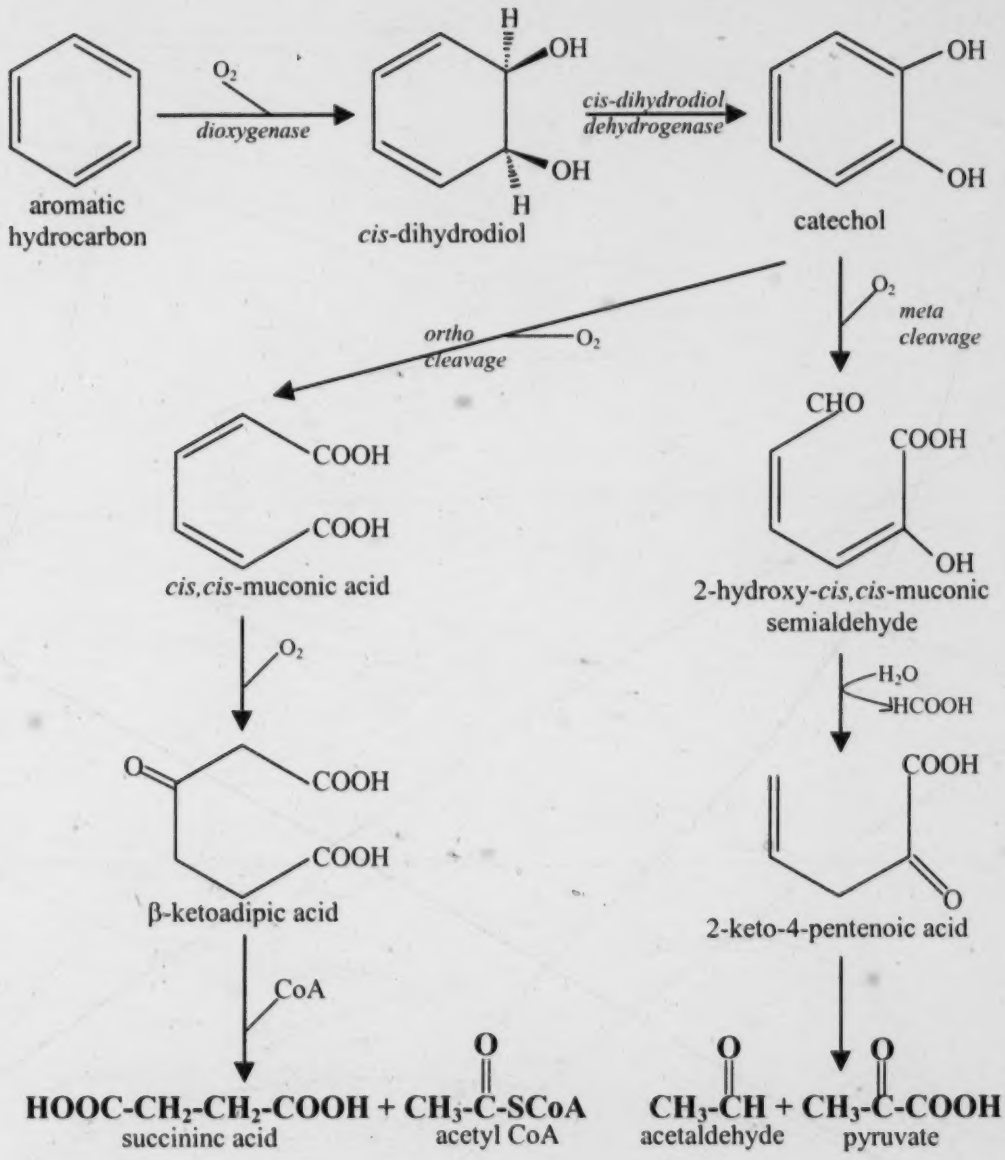

Figure 1.6. General scheme for oxidation and ring cleavage of aromatic hydrocarbons. (Cerniglia, 1984; Cerniglia, 1992).

compound is the oxidation of the alkyl substituent directly to form an alcohol, aldehyde, and carboxylic acid (benzoic acid) which is then oxidized to form a catechol.

Metabolism of PAHs by bacteria has been described for naphthalene, acenaphthene, acenaphthylene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, benz[a]anthracene, chrysene, and benzo[a]pyrene (Cerniglia, 1984; Cerniglia, 1992; Sutherland *et al.*, 1995). The specifics of the phenanthrene pathways are illustrated in Figure 1.7. Once phenanthrene is oxidized to 1-hydroxy-2-naphthoic acid, two pathways are known to exist. One parallels the naphthalene pathway, by the formation of 1,2-dihydroxynaphthalene and continued metabolism to catechol. The metabolism of catechol can then proceed as illustrated in Figure 1.6. This pathway has been described for *Pseudomonas* spp. (Sutherland *et al.*, 1995). The second phenanthrene pathway converts 1-hydroxy-2-naphthoic acid to *o*-phthalic acid and continues with the formation of protocatechuic acid. This phenanthrene pathway has been described for *Aeromonas* spp. and *Mycobacterium* spp. (Sutherland *et al.*, 1995).

Many fungi, some bacteria, and cyanobacteria attack the aromatic ring by the formation of an arene oxide and a *trans*-dihydrodiol, by incorporating one molecule of oxygen via cytochrome P_{450}-catalyzed monooxygenase and epoxide hydrolase reactions. Figure 1.8 outlines the general mechanism for fungal formation of a *trans*-dihydrodiol. Additionally, the arene oxides may undergo non-enzymatic rearrangements to form phenols. Fungal oxidation is known for naphthalene, acenaphthene, fluorene, anthracene, phenanthrene, fluoranthene, pyrene, benz[a]anthracene, and benzo[a]pyrene (Sutherland *et al.*, 1995). Fungal oxidation of PAHs plays a role in detoxification as opposed to catabolism and assimilation (Cerniglia, 1992). The oxidation of aromatic hydrocarbons by fungi is similar to their oxidation by mammals. Many of the arene oxides produced by fungi are potent carcinogens, whereas the *cis*-diols produced by bacteria are not (Sutherland *et al.*, 1995).

Some white-rot fungi, which decay lignin and wood cellulose, are able to oxidize PAHs without the formation of *cis*- or *trans*-dihydrodiols (Sutherland *et al.*, 1995). Quinones are one class of the major metabolites produced by these fungi. Mineralization and the production of polar metabolites has also been reported (Cerniglia, 1992). Figure

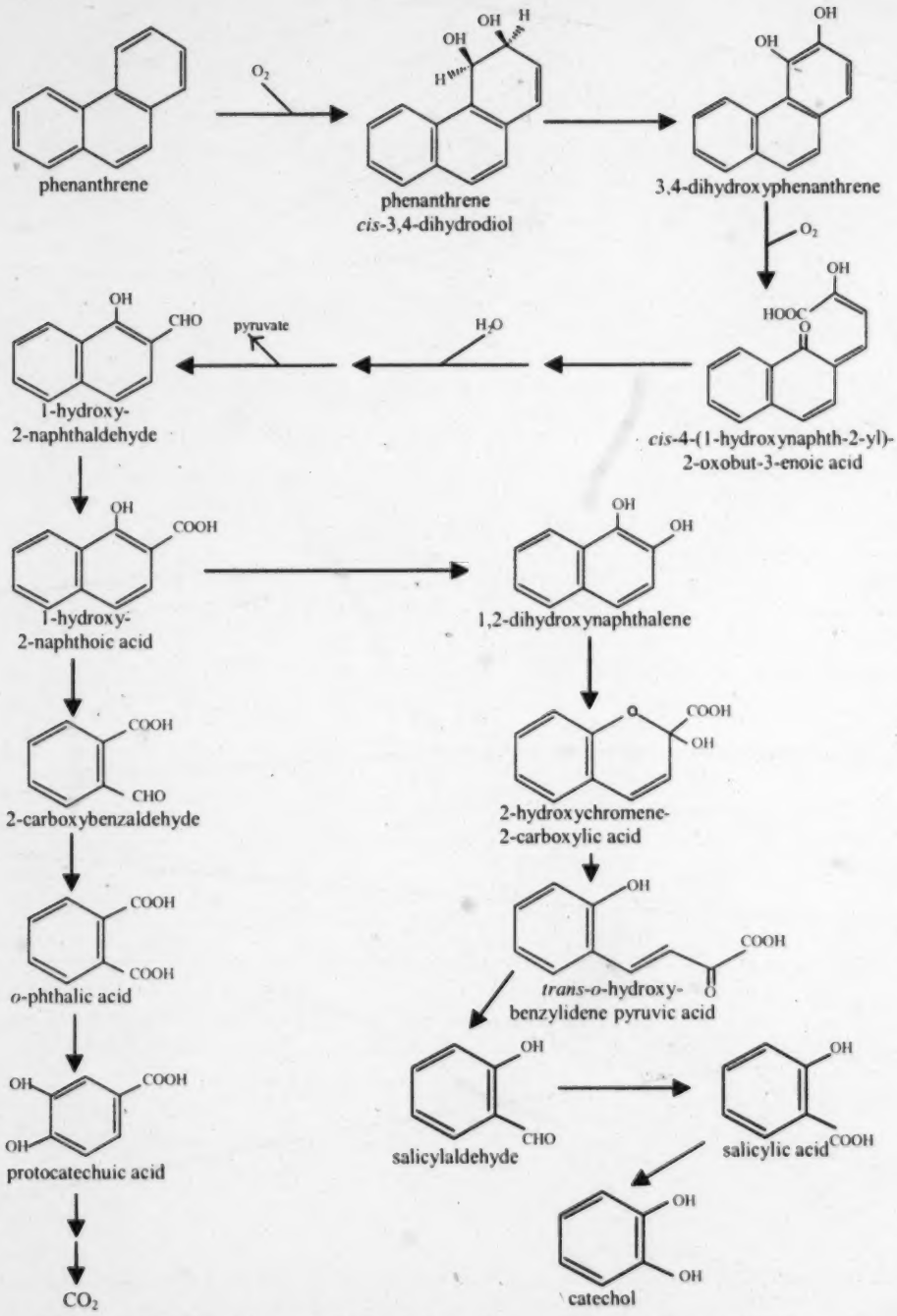

Figure 1.7. Bacterial metabolism of phenanthrene. (Sutherland *et al.*, 1995).

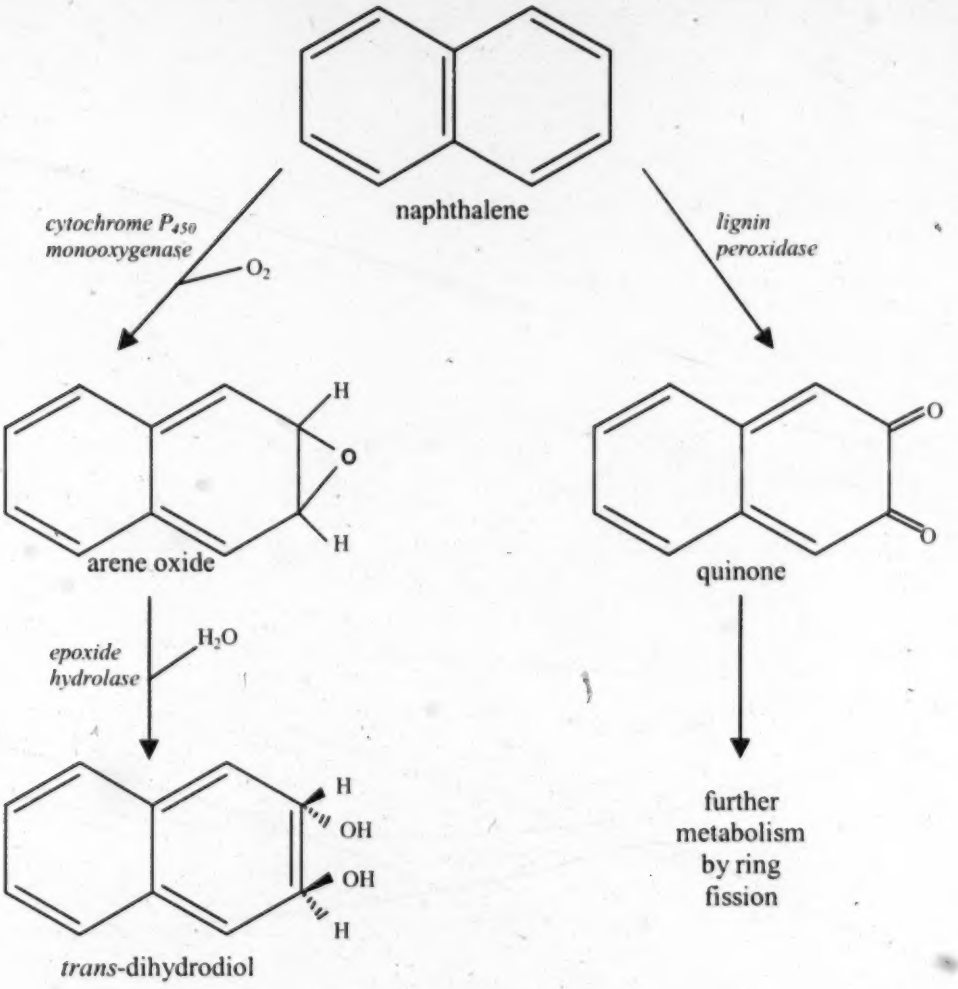

Figure 1.8. Fungal oxidation of naphthalene. Formation of *trans*-dihydrodiols by cytochrome P_{450} monooxygenases and epoxide hydrolases or quinone formation by lignin peroxidases of white rot fungi. (Cerniglia, 1992).

1.8 also illustrates the fungal oxidation of aromatic hydrocarbons by white-rot fungi. Lignin peroxidases represent the major class of enzymes involved in this type of oxidation.

1.3.3. Factors affecting biodegradation

In general the susceptibility of a compound to biodegradation depends on the type and size of the compound (Atlas, 1984). *n*-Alkanes of chain length *n*-C_{10} to *n*-C_{24} are degraded most rapidly whereas short-chain alkanes are toxic. Long chain alkanes are more resistant to biodegradation, however biodegradation of *n*-alkanes up to *n*-C_{44} has been reported (Atlas, 1981). Alkyl substitution of *n*-alkanes reduces the rate of biodegradation, as β-oxidation can be blocked by side chains requiring additional enzymatic activities (Atlas, 1981). Aromatic compounds generally are degraded more slowly than alkanes. High molecular weight aromatics, resins, and asphaltenes are considered to be recalcitrant (Atlas and Bartha, 1992). The genotoxicity and recalcitrance of PAHs generally increases as the molecular size of the PAH increases up to four and five fused benzene rings (Cerniglia, 1992). In addition, extensive alkyl substitution of PAHs can inhibit biodegradation. Alicyclic compounds are resistant to biodegradation unless a long side chain is present. Often degradation of alicyclic compounds requires cometabolism. In many cases the organism is utilizing another substrate as an energy source while cometabolizing the other substrate. In general the order of degradation is: *n*-alkanes > branched alkanes > low molecular weight aromatics > cyclic alkanes > high molecular weight aromatics or PAHs (Leahy and Colwell, 1990; Atlas, 1981).

Microorganisms capable of degrading crude oil are more likely to be found in regions where pollution is more extensive rather than in a pristine environment. However, the isolation of microorganisms capable of utilizing hydrocarbons is widespread, even from environments not subject to high levels of contamination (Atlas, 1981). Shifts in microbial populations have been described in response to the input of hydrocarbons (Leahy and Colwell, 1990) where an increase in both number and diversity of hydrocarbon-degrading organisms occurs. The percentage of hydrocarbon-utilizing microorganisms is a sensitive measure of the environmental exposure to contaminants.

For example, pristine ecosystems often support <0.1% hydrocarbon-utilizing organisms whereas contaminated sites can contain up to 100% hydrocarbon-utilizing organisms. Individual organisms can only degrade a limited number of hydrocarbon substrates, and therefore complete degradation of petroleum contaminants from a site, requires microbial consortia capable of degrading the full range of compounds comprising the contaminating oil or petroleum product (Leahy and Colwell, 1990). Many of the genes required for biodegradation are often contained within catabolic plasmids (Atlas and Bartha, 1992).

Atlas (1981) discussed diauxic growth of bacteria in which hexadecane degradation was reduced in the presence of acetate, and where pristane degradation did not occur in the presence of hexadecane. It was not known whether this form of sparing was due to catabolite repression. It has been suggested that this diauxic growth does not alter the biochemical pathways for degradation, but merely determines which pathways are expressed. This phenomenon plays an important role in the biological degradation of oil as well as influencing the type and extent of biodegradation within a contaminated site. Diauxic growth is an important consideration for bioremediation, as the type of biodegradation at a particular site will not only be influenced by the ability of a microorganism to degrade certain compounds but also the ability to degrade those compounds in the presence of other compounds.

The role of cometabolism is important as a wider variety of compounds can be degraded with a consortium than by an individual (Atlas, 1981). Organisms are able to oxidize some compounds while growing on others. The oxidized compounds could then serve as substrates for other organisms. Trzesicka-Mlynarz and Ward (1995) have shown that biodegradation of fluoranthene and other higher molecular weight PAHs was greater with a mixed culture of environmental isolates compared to biodegradation by these isolates individually. As many biodegradation studies are conducted with single isolates, it is important prior to *in situ* applications of bioremediation to understand how these isolates behave in the environment with other populations. A potential drawback to bioremediation is the conversion of certain compounds to oxidized products that are more persistent or potentially more toxic than the parent compound. Knowing the fate of incompletely degraded compounds is an important consideration.

The solubility of petroleum contaminants can greatly affect the ability of microorganisms to degrade them. Often contaminated sites contain compounds in concentrations in excess of their solubilities (Atlas, 1981) and degradation of high molecular weight compounds is dependent upon the dissolution of the compound such that biodegradation is limited by the bioavailability of the compound not the concentration (Leahy and Colwell, 1990). The surface area of the oil may also be another limiting factor. In marine spills the oil will spread as a thin slick which increases the available surface for colonization and degradation by petroleum-degrading organisms (Atlas and Bartha, 1992). However, in cases where extensive wave action occurs, the oil may become resistant to microbial attack as very large "mousse" emulsions form, which in turn decreases the volume to surface area ratio (Leahy and Colwell, 1990). Hydrocarbon-degrading microorganisms are known to act mainly at the oil-water interface (Atlas, 1981).

The use of chemical dispersants or surfactants can increase the rate (by increasing the surface area available) but not the overall extent of biodegradation. Use of such agents can increase the toxicity to higher organisms and therefore should be considered carefully prior to their use (Leahy and Colwell, 1990; Atlas, 1981). Extensive research has been conducted utilizing surfactants to increase the bioavailability of high molecular weight PAHs (Shuttleworth and Cerniglia, 1995). Research in this area has proven inconclusive. While some reports indicate that the inclusion of surfactants increases biodegradation (Cuny *et al.*, 1999) other studies indicate no increase in biodegradation and even an inhibitory effect to microorganisms. In a study by Allen *et al.* (1999) contradictory results were obtained with phenanthrene degradation and the inclusion of Triton X-100. Biodegradation was enhanced by the inclusion of this non-ionic detergent for *Pseudomonas* sp. strain 9816/11, while biodegradation by *Sphingomonas yanoikuyae* B8/36 was inhibited. The contradictory results obtained in these studies with the inclusion of biosurfactants for enhancing biodegradation, may be due to different responses of the organisms used to the various surfactants employed. The use of artificial surfactants to enhance bioremediation must be carefully evaluated in light of these conflicting studies. Many microorganisms produce biosurfactants, which may in turn increase the rate of biodegradation (Leahy and Colwell, 1990; Atlas and Bartha, 1992).

In cases of terrestrial petroleum contamination, the oil is not dispersed horizontally as in the case of marine spills, but rather vertically, down into the soil (unless the soil is frozen or waterlogged). This in turn decreases the evaporative losses of volatile compounds and through absorption of the contaminant to soil particles, reduces the bioavailability of the compounds (Leahy and Colwell, 1990; Atlas and Bartha, 1992). Due to infiltration of the contaminant into the soil, photooxidation is reduced. Another potential problem for bioremediation of terrestrial contaminated sites is the vertical movement of the oil down to the water table or underground aquifers. Further displacement by rainwater and fluctuations in the water table cause the contaminated region to spread. The degree of infiltration of oil into soil is dependent upon the porosity of the soil, water saturation of the soil, and viscosity of the oil. Solubilization of the contaminating hydrocarbons through the action of microbial oxidation further promotes the transport of contaminants within the soil (Bossert and Bartha, 1984).

The viscosity of oil is increased at low temperatures. This in turn decreases the amount of spreading of oil and thereby decreases the available surface area for microbial attack (Atlas, 1981). Low temperatures and ice cover reduce the volatilization of low molecular weight hydrocarbons, which are often toxic. Reduced volatilization of these compounds can delay the onset of biodegradation by increasing the lag time. Reduced temperatures are also known to decrease the rate of biodegradation due to lowered enzymatic activity (Leahy and Colwell, 1990). Biodegradation is known to occur over a wide range of temperatures by psychrotrophic, mesophilic, and thermophilic organisms, however the rates of degradation are reduced as temperature decreases (Atlas and Bartha, 1992). The temperature ranges in marine ecosystems is from $-2^{\circ}C$ to $33^{\circ}C$, whereas soil ecosystems can range from $-40^{\circ}C$ to $+65^{\circ}C$ (Bossert and Bartha, 1984). This extensive range in temperature poses unique challenges for biodegradation. For instance, freezing of water within soil limits growth of microorganisms.

The effects of limited nutrients is also known to impede biodegradation (Atlas, 1981), notably fixed nitrogen (N) and phosphorus (P) concentrations below the laboratory optimum ratio of 10:1 carbon (C) to N and 100:1 C to P. The input of hydrocarbons into an environment increases the C content greatly, which results in an overall reduction of the relative concentration of other nutrients. Numerous reviews cite cases where the

addition of fertilizers or N and P have enhanced rates and extents of biodegradation (Atlas, 1981; Atlas, 1984; Leahy and Colwell, 1990). However, the effect of adding nutrients on biodegradation is not uniform. Differences have been noted whereby saturated hydrocarbons are degraded more readily with the addition of nutrients while aromatic hydrocarbon degradation was not enhanced or even limited (Atlas, 1981). In addition, the effect of added nutrients may stimulate certain organisms such as bacteria but not fungi. Some of the cases reviewed in the literature indicate that biodegradation was inhibited by the addition of nutrients compared to control sites. The variability of the effect of nutrients on biodegradation has been attributed to the complexity of the systems and the fact that much more research is required in this area to elucidate these factors (Leahy and Colwell, 1990). Fayad and Overton (1995) noted that biodegradation of oil spilled during the Gulf War was unusual in that aromatic compounds were degraded more readily than saturated compounds. In addition to this, aromatic biodegradation was inhibited by the addition of nutrients while saturate compound biodegradation was enhanced. Wrenn *et al.* (1994) noted that biodegradation of light Arabian crude oil by an enrichment culture was inhibited by the addition of ammonium due to acidification, whereas amendment with nitrate did not inhibit biodegradation. Adequate buffering of the culture prevented these effects. Foght *et al.* (1999) noted acidification when ammonium, but not nitrate, was used to amend a mixed consortium of saturate and aromatic degraders grown on Alberta Sweet Mixed Blend crude oil. The acidification caused by saturate-degrading organisms inhibited aromatic degradation, but pH buffering minimized these effects. These studies indicate that the types of nutrient amendments must also be considered for applications in bioremediation.

The availability of oxygen is another factor that greatly influences biodegradation. Given that the initial steps in many of the biodegradation pathways involve the addition of oxygen via oxygenase enzymes, this would appear to be the rate-limiting step at reduced oxygen concentrations (Leahy and Colwell, 1990). Interest in the forced aeration of contaminated sites is increasing, however open seawater and other such systems would have to rely upon natural mechanisms for aeration such as wave action and turbulence. Anaerobic degradation of petroleum contaminants is known to occur but is often considered negligible compared to aerobic biodegradation (Leahy and Colwell, 1990).

Although the cumulative effect of anaerobic degradation may play an important role in bioremediation, it will not be considered here as this discussion is focussed on the aerobic conditions of biodegradation.

Increased salinity and pressure may also reduce the rates and extents of biodegradation (Atlas, 1981). This would cause petroleum contaminants to persist for longer periods of time in environments such as deep-sea sediments (Leahy and Colwell, 1990). Decreased rates of biodegradation under hypersaline environments may be due to an overall reduction in biological activity (Leahy and Colwell, 1990).

Biodegradation rates in terrestrial environments may be limited due to a reduction in water activity. Limiting water activity reduces microbial growth and metabolism and therefore leads to reduced rates of biodegradation of petroleum contamination in these environments (Leahy and Colwell, 1990). Conversely, high water activity in contaminated soils can reduce the oxygen concentration, again limiting biodegradation. The pH of terrestrial soils can also limit biodegradation. As soil pH can vary widely (pH 2.5 to 11.0; Bossert and Bartha, 1984) from the optimum for growth and metabolism of certain organisms, the ability to degrade contaminants varies.

Atlas (1981) cites many studies conducted on the natural biodegradation rates of petroleum contaminated sites. At many of these sites, biodegradation was limited by reduced temperature, oxygen availability and nutrient availability. Many of these studies reported losses of *n*-alkanes, but minimal removal of branched alkanes and aromatics. In these cases the indigenous microflora were capable of removing some of the contaminating substances without the addition of nutrients or seeding the sites with known degraders, but biodegradation was incomplete and often took years before the effects were observed.

To summarize, the factors affecting biodegradation and bioremediation of a contaminated site include:

a) the susceptibility of certain compounds to be degraded,

b) the presence and metabolic activity of organisms able to degrade or cometabolize compounds,

c) the preferential utilization of certain compounds over others,

d) bioavailability of the compounds to the microorganisms,

e) the presence of low molecular weight (toxic) compounds, and

f) abiotic factors such as limited nutrients, reduced oxygen availability, reduced temperature, low water activity, as well as extremes in salinity, pressure, and pH.

This list is not exhaustive, as each ecosystem is unique in its own complexity and interactions. Due to these complexities of marine, freshwater, and terrestrial ecosystems, one might expect that no one "formula" could be applied to a contaminated site with the hopes of achieving complete bioremediation.

Current approaches to stimulate biodegradation at contaminated sites involve the use of aeration, nutrient supplementation by the addition of fertilizers ("biostimulation"), and by seeding the contaminated site with known organisms capable of degrading the pollutant of interest ("bioaugmentation") (Atlas and Bartha, 1992). For these amendments to be effective, not only does the contaminant have to be degraded, but also the products of biodegradation should be non-toxic or at least less toxic than the parent compounds, and seeded organisms should be nonpathogenic with minimal adverse effects on the resident flora and fauna. Other approaches under consideration involve the use of synthetic and bio-dispersants to increase the bioavailability of the petroleum pollutant to the microorganisms. To increase the effectiveness of seeding, the use of genetically engineered organisms could be employed. These organisms would contain the necessary genes to carry out the degradation of a wider variety of compounds, however this type of research and its potential application has been areas of controversy (Atlas and Bartha, 1992). The effectiveness of seeding a contaminated site with either genetically altered or non-altered organisms is also debatable as hydrocarbon-degrading organisms appear to be ubiquitous throughout many environments and for a seeded organism to be effective, successful competition with the already established microflora would have to occur (Atlas, 1995).

1.3.4. Genetics of hydrocarbon degradation

Much is known regarding the genetics and regulation of the well-characterized metabolic pathways of hydrocarbon biodegradation. Many of the genes required for biodegradation are encoded on catabolic plasmids, which have been extensively reviewed

(Singer and Finnerty, 1984a; Yen and Serdar, 1988). The loci of the genes required for *n*-alkane oxidation have been mapped on the OCT plasmid encoded by *Pseudomonas putida* PpG6 (Singer and Finnerty, 1984a).

Other catabolic plasmids carrying genes for aromatic hydrocarbon degradation have been isolated and well characterized from various *Pseudomonas* spp. Plasmids for salicylate, toluene, and naphthalene degradation are known and well studied (Singer and Finnerty, 1984a; Yen and Serdar, 1988). The genetics of toluene and naphthalene biodegradation are well understood through the characterization of the TOL and NAH7 plasmids (Atlas and Bartha, 1992). Both plasmids contain dual operons for an upper and a lower pathway. In the NAH7 plasmid, the upper pathway converts naphthalene to salicylate while the lower pathway oxidizes salicylate, both pathways are induced by salicylate (Yen and Serdar, 1988). The TOL plasmid upper pathway converts toluene to benzoate and xylenes to toluates, which is induced by toluene. The lower pathway is responsible for *meta*-cleavage of benzoate and toluates and is induced by benzoate (Singer and Finnerty, 1984a).

Studies with these few substrates have greatly increased the knowledge and understanding of hydrocarbon biodegradation, and comparisons between the enzymes and regulation of these pathways can be used to determine unique biodegradation mechanisms. However, the genetics of metabolism of higher molecular weight PAHs are less understood and more research is required to reveal the genes required for these pathways and enzymes (Cerniglia, 1992).

1.4. Concerns for cold climate bioremediation

Biodegradation of PAHs under cold conditions is temperature-limited (Leahy and Colwell, 1990) as discussed in Section 1.3.3. This is due to the physical nature of the molecules (increased viscosity and decreased volatility of toxic compounds) as well as reduced rates of enzymatic activity (Atlas and Bartha, 1992). Typically, low nutrient levels in these cold environments also limit biodegradation. Although these factors play crucial roles in the extents and rates of biodegradation of cold climates, there are other specific factors and consequences that must also be considered in more extreme environments such as the Arctic, Antarctic, and sub-polar regions.

Arctic regions with short growing seasons and N-poor soils exhibit more prolonged and deleterious effects on plant life as a result of petroleum contamination (Bossert and Bartha, 1984). In addition, many of the food webs in Arctic and Antarctic environments are more tightly interconnected than temperate environments, increasing the risks of bioaccumulation. Cold stress within animals has been shown to increase the toxicity of various chemicals by reducing the concentration of detoxifying enzymes in the liver and by increasing the energy demands of organisms to maintain homeostasis (Robins *et al.*, 1999). Chemically induced hypothermia (body cooling to minimize the effect of a toxic chemical) also increases fatalities among animal populations by increased chemical exposure.

Many of the cold northern and southern environments are semi-arid and lacking in extensive vegetation cover. This condition increases the potential for aerial transport of contaminants, thereby increasing the contaminated area more than would be observed for similar contamination in more temperate environments. Previously, it was thought that frozen soil would create an impermeable barrier to hydrocarbon contaminants preventing vertical movement. Now, some studies have shown that vertical movement of liquid contaminants through air voids in frozen soils and capillary suction into small fissures in the frozen soil matrix can spread the contaminants and increase the extent of the problem (Biggar and Nahir, 1999).

For microorganisms to survive and proliferate in extreme environments and often under oligotrophic conditions, they must adapt specifically to that environment. These include conditions of low temperatures, dryness, extremes in salinity and pH, low nutrient availability and, in polar regions, seasons of continuously high solar radiation or darkness.

Vincent (1988) has described the strategies employed by microorganisms in order to overcome these extremes in environmental conditions. Colder temperatures generally reduce cellular activity by slowing the catalytic activity of enzymes. Conformational shifts in enzyme structure or synthesis of cold tolerant enzymes may maintain catalytic activity under colder conditions. Maintaining membrane fluidity under cold conditions is essential. Increasing the level of unsaturations, decreasing the acyl chain length, increasing the extent of branching, or decreasing the proportion of cyclic fatty acids

within the lipid bilayer, help to maintain fluidity at colder temperatures. Under freezing conditions cells can rapidly dehydrate as water is expelled from the cell to prevent intracellular ice formation, in favour of extracellular ice. Some of the survival strategies described include the secretion of substances to prevent ice nucleation in the vicinity of the cell, increased solute content to decrease the freezing point intracellularly, increased membrane water permeability to expunge water from the cell, changing the elasticity of the cell membrane to allow expansion during freezing and upon rehydration, and endospore formation.

Hypersaline environments, while decreasing the freezing point of water in cold environments, can negatively influence organisms trying to survive there, by dehydration due to high osmolarity of the environment, which in turn concentrates solutes within the cell and alters the water activity (Vincent, 1988). To survive these conditions, some microbial cells may accumulate compatible solutes, such as potassium chloride, which act to maintain a high internal osmolarity.

Phototrophic organisms increase the concentration of photosynthetic pigments during periods of continuous low light or utilize the concurrent cold temperatures to decrease cellular activity to survive during periods of complete darkness in polar regions (Vincent, 1988). During periods of continuous light in polar regions, microorganisms are exposed to increased solar radiation and with the reduction in ozone within the Antarctic, UV radiation is increased. Pigmentation of microorganisms, particularly with carotenoids, act as a protective agent against radiation damage (Krinsky, 1976).

In general, visible light acts to activate a photosensitizing molecule to an excited state. This can then either interact directly with a substrate or with oxygen to yield singlet oxygen which can then interact with a second substrate. UV radiation interacts directly with nucleic acids, activating them as a photosensitizer. Damage to a cell caused by these two forms of radiation include lethal and non-lethal mutations as well as the disruption of cellular respiration. Carotenoid pigments primarily protect the cell by transferring energy directly from singlet oxygen and dissipating it to the surrounding medium, but they can also interact with excited state photosensitizers and return them to ground state or react with the excited state singlet oxygen and remain oxidized (Krinsky, 1976). In addition to their protective effects against radiation, carotenoid pigments also

can make the cell membrane more rigid and have been implicated in the protection of cells against oxidative reactions when ingested by polymorphonuclear leukocytes.

1.5. Summary of existing literature on cold climate bioremediation

Biodegradation studies under cold conditions have received minimal attention in the literature in the past. However, in recent years, studies of this nature have received greater interest. As contamination within Arctic, Antarctic, and sub-polar regions increases due to an increase in human activity, effective measures for bioremediation are required. To accomplish this, research must be conducted to determine the presence of organisms capable of biodegradation under these cold and often limiting conditions. Despite the presence of microorganisms that are capable of degrading various hydrocarbon contaminants, rapid stimulation of biodegradation is often minimal. Factors limiting biodegradation (such as nutrient availability) must be understood to achieve successful *in situ* applications.

Many studies focus on the bioremediation ability of pre-existing and adapted microorganisms at a contaminated site. Whyte *et al.* (1999) assessed the bioremediation potential of petroleum hydrocarbon contaminated soils in the high arctic. Indigenous microorganisms were found with the ability to biodegrade hydrocarbons at the contaminated sites. During the summer months, nutrient supplementation with fertilizers markedly increased the rate and extent of biodegradation compared to untreated soils. Bioaugmentation with a consortium and with the addition of nutrients did not increase the overall extent of biodegradation. This study implies that the contaminated soils of the high Arctic most likely contain a sufficient quantity of cold-adapted, hydrocarbon-degrading microorganisms to achieve biodegradation with the supplement of nutrients alone. Aislabie *et al.* (1998) investigated a JP8 fuel contaminated soil in the Antarctic and found hydrocarbon-degrading microorganisms capable of degrading alkanes and aromatic hydrocarbons. N amendments were found to increase biodegradation. Other degradation studies under cold conditions have indicated that stimulation of indigenous organisms with the addition of nutrients is an effective means to increase biodegradation (Wilson *et al.*, 1999; Braddock and Walworth, 1999).

Despite the fact that many studies have focussed on the use of indigenous populations to assess biodegradation potential in cold environments, there have been some studies addressing the isolation and characterization of individual psychrotrophic and psychrophilic organisms with the ability to biodegrade under cold conditions. In a study by Whyte *et al.* (1998), alkane and diesel fuel biodegradation by the psychrotrophic organism *Rhodococcus* sp. Q15 was assessed. Short chain alkanes were found to be more readily degraded than longer chain alkanes.

Due to the short period of time in polar and sub-polar regions suitable for active biodegradation and bioremediation, optimal conditions for rapidly stimulating existing adapted populations is necessary. Similarly, bioaugmentation must use consortia able to overcome the short growing season with little to no lag time prior to commencing biodegradation, as well as successfully competing with the existing population.

1.6. Antarctica and the isolation of *Sphingomonas* sp. ANT 17

Sphingomonas sp. ANT 17 was isolated by J. Aislabie (LandCare Research, Hamilton, New Zealand). This section describes Antarctica in general and the physical and chemical characteristics of the isolation site of ANT 17.

1.6.1. General history, climate and biology of Antarctica

Antarctica is often described as the coldest, driest, and windiest place on Earth (Meadows *et al.*, 1994). A massive ice sheet roughly the size of Australia covers Antarctica, more than 4 km thick in places, leaving only 2% of the continent free of ice. Rock lies beneath the ice, and evidence exists that Antarctica was once temperate with extensive vegetation and reptilian fauna. Early interest in the continent was for exploration (to be the first to reach the South Pole and claim the continent for one's country!), as well as the sealing and whaling industries. The first documented arrival at the South Pole was by Roald Amundsen, who reached 90°S latitude on December 14, 1911.

By the late 1950s, scientific research stations were established at many locations along the Antarctic coast and sovereignty claims were made by Great Britain, New Zealand, Australia, Norway, France, Argentina, and Chile. The United States and the former Soviet Union reserved the right to make future claims. From July 1957 to

December 1958, international governance was initiated to accumulate knowledge of the region. By December 1, 1959, the Antarctic Treaty System was signed (and ratified 1961), by 12 participating nations. Currently, 26 nations participate in the policy and decision making procedures of Antarctica, while 15 other nations, including Canada, may participate in these meetings but do not hold the right to vote (National Research Council, 1993).

The Antarctic Treaty System governs all regions extending from 60°S latitude to the South Pole at 90°S latitude, which includes the continent of Antarctica, the surrounding islands and the encircling oceans (collectively referred to as the Southern Ocean). Three main principles define the treaty (National Research Council, 1993). Firstly, the region is to be a zone of peace, and the Treaty prohibits the establishment of military bases as well as weapons testing. Secondly, the Treaty acknowledges the importance of scientific research and promotes the free exchange of data and programs. The final principle of the Treaty puts the issue of territorial claims on hold.

Science dominates the stewardship of Antarctica. Scientific interest in the region includes meteorological observations, Earth's climatic history, astronomy, solar-terrestrial-physics, geological history, glacial history, biological research, and ecology. Despite the approach of stewardship over Antarctica, pollution and contamination of this otherwise pristine environment have occurred where humans have come into contact with the region, due to the establishment of research stations, construction of airstrips, as well as shipping lanes. Besides scientific pursuits, tourism also has the potential to negatively impact the region. Commercial interest has been focussed on the exploitation of Antarctica's mineral and hydrocarbon reserves (St. John, 1990). Not until 1991 did the Protocol on Environment Protection to the Antarctica Treaty come into force outlining the protocols and procedures for all activities with potential for environmental impact. A portion of this Protocol involves the limitation of foreign organisms in the use of bioremediation, i.e. all organisms used for this purpose must originate from Antarctica.

Friedmann (1993) and Vincent (1988) have compiled information regarding the microbiology and microbial ecosystems of Antarctica. In general, the vast majority of the living indigenous organisms are composed of microorganisms. The Antarctic ecosystems include the sea-ice, ice shelves, benthic, marine, glacial, soil, flowing water

and lake, as well as lithophytic (rock) ecosystems. Protozoa, fungi, bacteria, and microalgae dominate these ecosystems. As the continent is lacking in higher plants, the majority of the primary production in Antarctica is by cyanobacteria and microalgae.

The soil ecosystems in Antarctica (both coastal and inland) pose unique challenges for microorganisms. Most of the soils are cold, dry and of high salinity and are subject to rapid freeze-thaw cycles (Vincent, 1988). Cold temperatures in these soils limit both biological and chemical weathering of the soil as well as biological activity. Antarctic soils pass through the freezing point several times a day. Approximately 90% of these ice-free soils are subject to snowfall, which provides moisture for microorganisms, but further limits soil heating and microbial processes. These soils have an unusually high salt content, consisting of calcite, gypsum, chlorides, nitrates, and sulphates. This is typical of both hot and cold deserts where evaporation exceeds precipitation and where the soils are not flushed with meltwater or streams. Salts enter the soil from sea spray, penguin nasal secretions as well as penguin guano. The pH of Antarctic soils varies widely from pH 3.5 to 9.0. The low clay and organic content of Antarctic soils limits its buffering capacity. Cyanobacteria, mosses, and lichens dominate as the phototrophic communities in Antarctic soils. These soils also contain numerous bacterial, fungal, and protozoan species.

1.6.2. ANT 17 Isolation

The Ross Dependency of Antarctica, over which New Zealand has stewardship, contains an ice shelf (Ross Ice Shelf also known as the McMurdo Ice Shelf), and has soil and ice-free regions (Ross Desert; McMurdo Dry Valleys). The ice shelf is permanent, floating glacial ice (which is >400 m in thickness) and covers the southern potion of the Ross Sea. The Ross Desert consists of the year round ice-free regions of Antarctica with soils containing less than 0.3% moisture. The Ross Dependency, with the shortest direct route to the South Pole and numerous research facilities and proximity to the Dry Valleys, has been a region of Antarctica experiencing intense human activity. The type of impact in this region has included oil and petroleum contamination from drilling operations, vehicle, ship and aircraft movements, nuclear power generation, sewage and

waste disposal, as well as the aerial dispersal of particulate matter (fiberglass, cardboard and wood fragments).

ANT 17 was isolated from a soil site at Scott Base, Antarctica as a microorganism capable of growth on 1-methylnaphthalene but not naphthalene (Aislabie *et al.*, 1999). Isolation was directly from oil-contaminated soil, as soil dilutions were spread directly onto solidified Bushnell-Haas medium with 1-methylnaphthalene vapours as the sole C source. The isolation of ANT 17 was at 16°C. Scott Base is located on Ross Island in the Ross Dependency, surrounded by the ice shelf and the Ross Sea, and is covered in pack-ice during the winter. Scott Base is approximately at 78°S latitude and 167°E longitude. Appendix A.1 illustrates the geographical location of both the Ross Dependency within Antarctica as well as the location of Scott Base. Appendix A.2 illustrates the layout of Scott Base. ANT 17 was isolated from soil below the diesel tanks. This soil was subject to chronic leakage of JP8 fuel (similar to diesel), which contains *n*-alkanes from *n*-C_9 to *n*-C_{14}, as well as a few mono- and di-aromatics.

Appendix A.3.1 lists the quantities and types of hydrocarbon contaminants found at the ANT 17 isolation site as well as other contaminated sites at Scott Base. Specifically, the PAH content of the soil where ANT 17 was isolated is given. From these data, two- and three-ring aromatics are most common with trace amounts of larger PAHs. The total hydrocarbon data are compiled from the content of contaminants at other contaminated sites within Scott Base. This illustrates that the vast majority of the contamination is of *n*-alkanes from *n*-C_9 to *n*-C_{14}, as well as the mono- and di-aromatics. This directly reflects the composition of JP8, which is used for multiple purposes at the base.

Appendix A.3.2 compares the total percent hydrocarbon (TPH) found in contaminated soils to the amount of total heterotrophic bacteria and hydrocarbon-degrading bacteria enumerated in those soils. These data are compared to an uncontaminated control site away from vehicle tracks. Data are provided for different depths of the soil horizon. From these data, it is shown that as one would expect, hydrocarbon-degrading microorganisms are found in contaminated sites in a higher proportion than heterotrophs. This parallels the generalizations discussed in Section 1.3.3.

Appendix A.3.3 lists the general chemistry of the soil at various sites around Scott Base. From these data, the soil can be classified as dry and alkaline. The available N is low, whereas P is high. The conductivity measurements indicate that the soil is of low salinity. The temperatures of these soils in the summer are at –2°C in subsurface layers to +14°C on the surface while the air temperature is average –4°C. There is not much organic material within these soils to retain the hydrocarbons, and this should positively affect the bioavailability of the contaminants. The JP8-contaminated sites have abundant alkane degraders due to the high concentration of alkanes in JP8. These were predominantly Gram-positive isolates whose cell wall would allow them to survive in dry and alkaline soil. Gram-negative microorganisms were the main aromatic degraders (Aislabie *et al.*, 1999).

1.7. Summary of existing literature of *Sphingomonas* spp.

1.7.1. History and taxonomy

Pseudomonas paucimobilis was first named and described by Holmes *et al.* (1977) as a Gram negative rod, with yellow pigmentation and a polar monotrichous flagellum. The genus *Sphingomonas* was proposed by Yabuuchi *et al.* (1990) due to the similarity among *P. paucimobilis*, *Flavobacterium devorans*, and *Flavobacterium capsulatum* (based on DNA:rRNA hybridizations; Bauwens and De Lay, 1981). A taxonomic comparison of 16 clinically isolated strains included phenotypic characteristics, assimilation of various carbon sources, susceptibility to antimicrobial agents, cellular lipid and fatty acid analyses, determination of isoprenoid quinones, 16S rRNA analysis, G+C content, and DNA-DNA hybridizations. Microorganisms belonging to the genus *Sphingomonas* were classified on the basis of their 16S rRNA sequence, the presence of unique sphingoglycolipids and the major type of ubiquinone present (Q10). Three new species (*S. parapaucimobilis, S. yanoikuyae*, and *S. adhaesiva*) and one new combination (*S. capsulata*) were described. The type species is *Sphingomonas paucimobilis* (Holmes *et al.*, 1977) comb. nov. with the type strain NCTC 11030.

Yabuuchi *et al.* (1990) described *Sphingomonas paucimobilis* as deeply yellow pigmented and strictly aerobic, Gram-negative rods producing catalase. The strains tested in that study had a single polar flagellum when motile. All *S. paucimobilis* strains

had DNA in the range of 62% to 64% G + C. The main yellow pigment was determined by Jenkins *et al.* (1979) to be the carotenoid (2*R*, 3*R*, 2'*R*, 3'*R*)-β,β-carotene-2,3,2',3'-tetrol (nostoxanthin). Figure 1.9 shows the structure of this pigment. Nostoxanthin was previously only found in cyanobacteria, and the presence in *S. paucimobilis* was seen as a potentially unique taxonomic marker. Many of the early isolates of *S. paucimobilis* were thought to be *Xanthomonas* spp., but these microorganisms contain xanthomonadin, taxonomically distinguishing them from *Sphingomonas* spp.

Yabuuchi *et al.* (1990) reported the major fatty acids of the cellular lipids of this genus as including *cis*-octadecanoic acid (44% to 63 %) and 2-hydroxymyristic acid (18% to 31%). In addition to the three major cellular lipids (cardiolipin, phosphatidyl ethanolamine, and phosphatidyl glycerol), the cellular lipids were found to contain one major and 3 minor sphingoglycolipids, with the major one identified as glucuronosyl ceramide (with sugar moiety glucuronic acid, fatty acid 2-hydroxymyristic and the major long chain base as d-18:0; 59%). The presence of this sphingoglycolipid was deemed an important chemical criterion for determining the taxonomic position of *Sphingomonas* spp. Sphingoglycolipids are known within eukaryotic cell membranes, but previously not within prokaryotes. In addition, glucuronosyl ceramide is unique as this glycolipid has not been found in eukaryotes (Yamamoto *et al.*, 1978). Therefore the occurrence of glucuronosyl ceramide is unique and taxonomically distinguishing.

Kawahara *et al.* (1991) elucidated the chemical structure of sphingoglycolipids from *S. paucimobilis*. Based on previous observations that extraction of typical LPS was not possible from type strains of this genus and that extracted glycolipids lacked 3-hydroxylated (long chain) fatty acids, 3-deoxy-D-*manno*-2-octulosonic acid (KDO) and L-*glycero*-D-*manno*-heptose (L,D-Hep) typical of LPS, a new role for sphingoglycolipids was proposed. The occurrence of 2-hydroxymyristic acid was in the expected proportion of 3-hydroxylated fatty acids of lipid A, suggesting that *S. paucimobilis* possessed an unusual lipid A. The lipid A type lipid was demonstrated to contain equimolar amounts of *erythro*-1,3-dihydroxy-2-amino-octadecane and *erythro*-1,3-dihydroxy-2-amino-*cis*-13,14-methyleneoctadecane, linked to 2-hydroxymyristic acid. The oligosaccharide portion consisted of a tetrasaccharide (Man-Gal-GlcN-GlcA) or a monosaccharide (GlcA). The outer membrane of *Sphingomonas* spp. is thought to be composed of

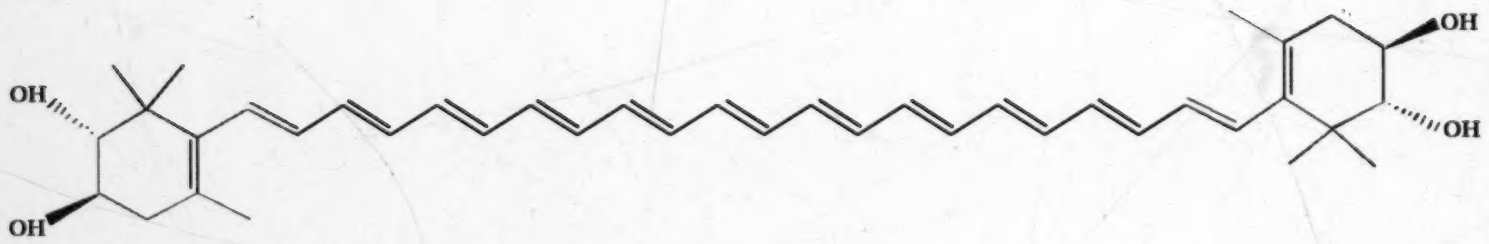

Figure 1.9. Chemical structure of the carotenoid pigment nostoxanthin. (Buchecker, *et al.*, 1976).

phospholipids and sphingoglycolipids with the latter assuming a role similar to LPS, in particular the R-type LPS.

Many *Sphingomonas* spp. have been identified and isolated as opportunistic pathogens. Miyazaki *et al.* (1995) investigated the pathogenic role of sphingoglycolipids. Acidic sphingoglycolipids were found to promote phagocytosis and phagosome-lysosome fusion by polymorphonuclear leukocytes. Wiese *et al.* (1996) investigated the role of sphingoglycolipids compared to LPS as a component of an asymmetric bilayer (composed of phospholipids on one side). Membrane potential, porin function and compliment activation were investigated. The antigenic surface structures and contributions to the functions of the outer membrane were found to be quite similar between these two glycolipids. One slight, yet notable difference, was in the investigation of fluidity of the bilayer with respect to presence of either glycosphingolipid or LPS. The bilayer containing LPS maintained a highly ordered gel phase from 10° to 30°C, with a sharp phase transition state at 31°C. However, the bilayer containing sphingoglycolipids exhibited a continuously decreasing state of order over the temperature range of 10° to 60°C. In the lower temperature range, the sphingoglycolipid bilayer was less ordered than the LPS bilayer. At 37°C, both asymmetric bilayers were of identical order in phase.

Pollock (1993) investigated *Pseudomonas*, *Alcaligenes*, *Azotobacter*, *Xanthobacter*, and *Xanthomonas* that secrete acidic heteropolysaccharides related to gellan. These strains were compared to *Sphingomonas paucimobilis* type strain ATCC 29837 (and an *Escherichia coli* negative control) due to their similarities as yellow, non-fermentative Gram-negative bacteria. Isolates from within the genera tested were shown to be closely related to the genus *Sphingomonas* based on various taxonomic tests and fatty acid analysis (compared to Yabuuchi *et al.*, 1990). The production of gellan by *Sphingomonas* spp. is of commercial, industrial, and biomedical interest (Manna *et al.*, 1996). Ashtaputre and Shah (1995) have demonstrated that the exopolysaccharides produced by *S. paucimobilis* isolate GS1, were in highest concentration with the inclusion of nitrate and glutamate compared to ammonium and urea, and this was not a pH dependent effect. Also, production was best at low N concentration. These exopolysaccharides have exhibited emulsifying properties with hydrocarbons. Recently,

Yamazaki *et al.* (1996) have cloned and sequenced the genes responsible for the synthesis of these capsular polysaccharides in *Sphingomonas* strain S88, collectively called sphingans, consisting of gellan, welan, rhamsan, and others. A 29 kbp sequence containing a four-gene operon was determined to be responsible for the synthesis of sphingan S-88. These gene products were related to those of other polysaccharide-secreting bacteria. Also within this region, two genes were found to encode for a lytic or toxic protein, suggesting that, like capsule production, sphingan synthesis is a defensive mechanism while the toxin is offensive in a pathogenic manner.

Another unique characteristic of the cell surface of *Sphingomonas* spp. has been suggested by Hasino *et al.* (1996). Electron microscopic analysis of the cell surface of an environmental isolate revealed the presence of large pits (0.02 to 0.1 μm in diameter) covering the cell surface, located in regions were the cell membrane appeared to sink into the cytosol. The function of these pits might be to facilitate direct uptake of large macromolecules, possibly through a mechanism similar to endocystosis. This function was suggested on the basis of a lack of extracellular and periplasmic enzymes for depolymerization of alginate. All of these enzymes were located within the cytosol exclusively. Furthermore, the presence of these pits was dependent upon the presence of alginate as a carbon and energy source. These pits might also be responsible for transport of other hydrophobic and large hydrophilic molecules across the cell wall and through the cell membrane.

Sphingomonas sp. strain RB2256, isolated from a marine sample in Alaska, has been shown to exhibit a unique growth characteristic (Fegatella *et al.*, 1998). This oligotrophic ultramicrobacterium exhibits a constant growth rate of 0.13 to 0.16 h^{-1}, regardless of glucose concentration and in the absence of a lag phase upon nutrient upshift, suggesting that ribosome content was not limiting, despite the finding that this isolate only possessed one copy of the rRNA operon on its genome. The growth rate of this isolate was slow compared to other heterotrophic marine bacteria.

The recent overview by Kersters *et al.* (1997) of the changes in the classification of Pseudomonads, confirms the placement of the genus *Sphingomonas* in the alpha subclass of the *Proteobacteria*. This review compiled data utilizing published rRNA analyses, and reconstructed the phylogeny of *Proteobacteria*. The genus *Sphingomonas* will most

likely continue to add new isolates and species as organisms are discovered or re-classified based on similar phenotypic characteristics.

1.7.2. Biodegradation ability

While many of the strains first identified as *Pseudomonas paucimobilis* (later *Sphingomonas*) were isolated from clinical and medical specimens, these organisms are continually isolated from the environment. In particular, many of these isolates were obtained from contaminated sites with the potential ability to degrade various petroleum related compounds and other pollutants. Several *Sphingomonas* spp. have been well characterized in recent years regarding their ability to biodegrade a variety of compounds, the pathways utilized or metabolites produced and the genetics of biodegradation that some of these isolates employ. The following discussion will highlight some of these *Sphingomonas* isolates. Table 1.1 summarizes some of the various isolates discussed, their sources, and the compounds these isolates are able to degrade. This table was compiled from research papers that have identified the respective isolates as *Sphingomonas* spp. or *Pseudomonas paucimobilis*.

Many studies have isolated a variety of *Sphingomonas* spp. with the ability to degrade aromatic compounds and some have identified metabolites and elucidated pathways. *S. paucimobilis* BA2 was isolated by Kastner *et al.* (1994) with the ability to grow on anthracene as a sole carbon source. S. *paucimobilis* isolate EPA505 was isolated by Mueller *et al.* (1990) because of its ability to degrade fluoranthene and a variety of other compounds. EPA505 was used in a study to determine the potential for biodegradation of four-, five- and six-membered compounds (Ye *et al.*, 1996) and was found to mineralize chrysene and benz[a]anthracene while transforming pyrene, benzo[a]pyrene, benzo[b]fluoranthene, dibenz[a,h]anthracene and 1-nitropyrene. Transformation and mineralization of these large and recalcitrant PAHs is a significant finding, as biodegradation of these compounds is rarely reported in the literature. *S. paucimobilis* isolate 2322 was utilized by Dutta *et al.* (1998) to investigate and elucidate the pathways used for biodegradation of methyl-substituted naphthalenes. Deep subsurface *Sphingomonas* isolates were compared to *Sphingomonas* isolate F199 for the ability to degrade various aromatic compounds (Fredrickson *et al.*, 1995). Four of the

Table 1.1. *Sphingomonas* spp. isolates with the capability to biodegrade various compounds.

Isolate	Compounds reported to be degraded	Source	Reference
S. paucimobilis ANT 17	See Table 3.5.	JP8 fuel contaminated soil, Scott Base, Antarctica	Aislabie *et al.*, 1998, and this study
S. (formerly *Pseudomonas*) *paucimobilis* WW3	dibenzothiophene, phenanthrene, fluorene, naphthalene, 1-methylnaphthalene	soil enrichment culture	Weissenfels *et al.*, 1990
S. (formerly *Pseudomonas*) *paucimobilis* EPA505	fluoranthene, naphthalene, 1- and 2-methylnaphthalene, 2,3-dimethylnaphthalene, phenanthrene, anthracene, benzo[b]fluorene, biphenyl, fluorene, anthraquinone, pyrene, and chrysene	creosote contaminated soil, Pensacola, FL	Mueller *et al.*, 1990
S. (formerly *Pseudomonas*) *paucimobilis* Q1	biphenyl, polychlorinated bipenyls, methylbiphenyls, hydroxybiphenyls, *m*- and *p*-xylene, toluene, *m*-methylbenzyl alcohol, *m*-tolualdehyde, benzoate, *m*- and *p*-toluate, salicylate, 4-hydroxybenzoate, and octane	soil, Chicago, IL	Furukawa *et al*, 1983
S. paucimobilis BA2	naphthalene, phenanthrene, and anthracene	tar-oil contaminated soil, Hamburg, Germany	Kastner *et al.*, 1994
S. paucimobilis 2322	methyl- and dimethylnaphthalenes, biphenyl, *o*-xylene, *p*-cymene, acenaphthene, and phenanthrene	cresosote contaminated soil	P. Chapman collection, US EPA (Dutta *et al.*, 1998)
S. paucimobilis SYK-6	5,5'-dehydrodivanillic acid (DDVA), syringate, vanillate, other dimeric compounds	pulp-bleaching wastewater	Katayama *et al.*, 1988
S. (formerly *Beijerinckia* sp.) *yanoikuyae* B1	biphenyl, naphthalene, *m*-xylene	stream, Austin, TX	Gibson *et al.*, 1973
S. aromaticivorans F199	toluene, naphthalene, fluorene, biphenyl, dibenzothiophene, salicylate, benzoate, *p*-cresol, and all xylene isomers	Southeast Coastal Plain subsurface sediments (407 m below land surface)	Fredrickson *et al.*, 1991

Table 1.1. (continued)

Sphingomonas sp. SS3	diphenyl ether, catechol, 4-fluoro-, 4-chloro-, and 4-bromo- diphenyl ether and catechol, phenol, 4-fluorophenol, benzoate, and 4-hydroxybenzoate	industrially contaminated soil, Germany	Schmidt *et al.*, 1992
Sphingomonas sp. RW1	dibenzo-*p*-dioxin, dibenzofuran	Elbe river water sample, downstream from Hamburg, Germany	Wittich *et al.*, 1992
Sphingomonas sp. HH69	dibenzofuran, dibenzo-*p*-dioxin, 9-fluorenone, dibenzothiophene, biphenyls, 2-, 3- and 4-acetodibenzofuran, 2-, 3- and 4-hydroxydibenzofuran,	soil enrichment culture, farmland and forest soil mixture	Fortnagel *et al.*, 1990
Sphingomonas sp. CF06	carbofuran, carbofuran-7-phenol, carbaryl, gentisate, protocatechuic acid	carbofuran exposed soil, western Washington	Feng *et al.*, 1997

five isolates were determined to possess the ability to utilize and mineralize a variety of compounds. Isolate F199 was later classified as a new species (*Sphingomonas aromaticivorans*) and some of the other deep-subsurface isolates were assigned to *Sphingomonas subterranea* and *Sphingomonas stygia* based on phylogenetic analyses (Balkwill *et al.*, 1997). It would appear that the ability of members of this genus to biodegrade aromatic compounds is widespread.

Other compounds and xenobiotics are known to be degraded by members of this genus. Schmidt *et al.* (1992) describe *Sphingomonas* sp. isolate SS3 capable of mineralizing diphenyl ether and its monohalogenated derivatives. Based on isolated metabolites, a pathway was proposed. Harms *et al.* (1995) studied *Sphingomonas* sp. isolate HH69 which was capable of degrading several substituted dibenzofurans. Isolate HH69 displayed significant regioselectivity, as specific substituted dibenzofurans yielded correspondingly substituted salicylic acids. *Sphingomonas* sp. RW1 is another isolate with the ability to degrade heterocycles (Wittich *et al.*, 1992). Recent studies with this isolate have analyzed the extradiol dioxygenase 2,2',3-trihydroxybiphenyl dioxygenase (Bertini *et al.*, 1995) and the genetic characterization of dioxin dioxygenase (Armenguad *et al.*, 1998). Surprisingly, the genes for this latter enzyme were scattered on the genome and not organized into typical operons. In addition, some of the genes were constitutive while others were regulated by the presence of an available carbon source. Similar results were observed Zylstra and Kim (1997) with *S. yanoikuyae* B1, where this isolate displayed broad substrate diversity which was induced by the presence of one of a variety of substrates. Six operons were noted with combinations of classes of enzymes, not restricted to one pathway, thus enabling a broad substrate diversity. Studies by Ka *et al.* (1994a, 1994b, 1994c) described numerous *Sphingomonas* isolates with the ability to degrade 2,4-dichlorophenoxyacetic acid (2,4-D), a herbicide. *Sphingomonas* spp. have also been isolated with the ability to degrade γ-hexachlorocyclohexane, an insecticide (Senoo *et al.*, 1996; Adhya *et al.*, 1996). *S. paucimobilis* isolate SYK-6 is able to grow on and degrade various compounds used as models for lignin degradation. Nishkawa *et al.* (1998) have cloned and sequenced a gene involved in O-demethylation of key intermediates in lignin degradation.

Many of the genes required for biodegradation of various compounds are plasmid encoded. Feng *et al.* (1997) describe a *Sphingomonas* sp. strain CF06 capable of mineralization of both the carbonyl group and the aromatic ring of the insecticide carbofuran. Five plasmids were identified in this strain and were required for growth on carbofuran as a sole C source. A study by Kim *et al.* (1996) cross-hybridized cloned genes for aromatic hydrocarbon (biphenyl and *m*-xylene) degradation from *S. yanoikuyae* B1 to surface and deep-subsurface *Sphingomonas* isolates. Genes cross-hybridizing to the two surface isolates were located on the chromosome, while the cross-hybridizing genes of the five deep-subsurface isolates were located on plasmids. It was suggested that the subsurface isolates may be ancestors of surface isolates.

Most recently, the entire 184-kb plasmid (pNL1) from *S. aromaticivorans* F199 was sequenced (Romine *et al.*, 1999). Of the 186 open reading frames described, it was determined that 79 of them likely encode enzymes for catabolism and transport of aromatic compounds. Fifteen clusters of genes for aromatic degradation have been determined. The authors noted that the appearance of genes from different pathways clustered together may be a response to similarities in the biochemical mechanisms for degradation of intermediates. In addition, putative transport genes have been identified. This sequence should serve as an excellent source for comparison with other *Sphingomonas* spp. sequences in order to determine inter- and intragenic similarities.

S. (formerly *Pseudomonas*) *paucimobilis* strain Q1 was investigated for common induction and regulation of biphenyl, xylene, toluene and salicylate catabolism (Furukawa *et al.*, 1983). Table 1.1 lists the compounds this strain was able to use as sole C and energy sources. By identifying major metabolites and comparing oxidation of biphenyl, xylene, toluene and salicylate after growth of Q1 on various substrates, it was determined that a common pathway governed the catabolism of these substrates. Benzoate and toluate were the common intermediates of growth on biphenyl, xylene or toluene. Salicylate was produced as a metabolite from 2-hydroxybiphenyl. Growth on these substrates induced oxidation of the *meta*-cleavage enzymes of both 2,3-dihydroxybiphenyl and catechol. Growth on benzoate resulted in the induction of the *ortho* pathway enzymes only. This induction scheme was shown to differ from the TOL plasmid enzymes as well as the SAL plasmid enzymes. Hybridizing the genome and the

three plasmids of Q1 to the TOL and SAL plasmids showed no significant homology. This suggested that the *meta*-cleavage enzymes of Q1 might be significantly different than those of the TOL and SAL plasmids. Taira *et al.* (1988) further characterized the 2,3-dihydroxybiphenyl dioxygenase gene from isolate Q1. This enzyme was determined to be functionally different than the previously studied 2,3-dihydroxybiphenyl dioxygenase from *Pseudomonas pseudoalcaligenes* KF707, despite empirical similarities. The enzyme from Q1 can utilize catechol while the KF707 enzyme can not, thus differing from catechol 2,3-dioxygenase.

From these studies and the results of many others with *Sphingomonas* isolates from often contaminated environmental sources, a pattern is emerging. Species of this genus appear to be ubiquitous, in deep-subsurface samples, soils, freshwater, and marine samples. Other isolates have been cultured from clinical and medical sources. Many of the studies with the environmental isolates describe broad ranges of substrates that these organisms can degrade, particularly aromatic and related compounds. Other than for *S. paucimobilis* isolate Q1, there has been no further mention of these isolates degrading *n*-alkanes or other saturated compounds (Table 1.1). Many of the isolates are capable of degrading a variety of compounds with no specific pattern regarding size or conformation. More recent work analyzing the genetics of *Sphingomonas* spp. biodegradation has and should continue to yield interesting results. Many of the genes and enzymes isolated from this genus are unique or significantly different from previously characterized enzymes from *Pseudomonas* and other well established degrading species. The mapping of various genes for degradation have also shown that members of this genus are less well evolved and regulated with respect to genetic organization, however, the dispersal of catabolic genes may indicate greater catabolic diversity, as has been seen with the compound studies. More research with isolates from this genus should prove to be interesting and enlightening with respect to the mechanisms and potentials for biodegradation.

1.8. Objectives

The purpose of this thesis was to characterize Antarctic isolate ANT 17.

a) As ANT 17 is a new isolate, the first objective was to define the organism taxonomically. This portion of the research included:

   i) classifying ANT 17 to the genus and species level using conventional microbiological techniques, fatty acid methyl ester (FAME) analysis and 16S rDNA sequence analysis,

   ii) describing ANT 17 according to its growth characteristics on complex medium under various conditions of temperature and pH,

   iii) determining whether ANT 17 contained plasmids or demonstrated metal resistance, UV resistance or other unique characteristics such as growth response to inoculum density; and

   iv) comparing some of these characteristics to similar species isolated from temperate environments.

b) Because ANT 17 was isolated from a JP8 jet fuel contaminated site in Antarctica, various petroleum-related compounds and their potential metabolites were screened qualitatively for the ability to serve as a C and energy source, or for susceptibility to transformation by ANT 17. Various methods were used, including liquid medium conditions, vapour, spray and overlay plating techniques.

c) ANT 17 was assessed for the ability to grow on and degrade several crude oils and refined oil products under cold and growth-limiting conditions. This study was conducted at temperatures ranging from 8° to 30°C, with and without N and P amendments, as well as under shaking or static culture conditions. Standard laboratory techniques were used, including dichloromethane (DCM) extraction, silica gel column fractionation and gas chromatography with flame-ionization detector (GC-FID) analysis.

d) The ability of ANT 17 to mineralize various compounds was also assessed. Standard techniques involved incubation with ^{14}C-radiolabelled compounds, trapping and counting radiolabelled $^{14}CO_2$, as well as quantifying the radiolabel recovered in the form of metabolites, parent compound and biomass, with the ultimate goal of completing a radiolabel distribution analysis post-incubation.

2. MATERIALS AND METHODS

2.1. Media and solutions

2.1.1. General culture media

Plate count agar (PCA; Difco, MI), Trypticase Soy broth (TSB; Difco or BBL, MD), and quarter-strength TSB (QTSB), Nutrient agar plus 0.5% glucose (NAG; Mueller *et al.*, 1990), Nutrient broth plus 0.5% glucose (NBG) and Luria Bertani agar (LB) were used for the general culturing of ANT 17 to assess purity of experimental cultures, to prepare suitable cultures as inocula, as well as for general growth observations. Appendix B.1 lists the components of NAG, NBG and LB.

2.1.2. Differential media and media for taxonomic determinations

KF agar, McConkey's agar, Eosin Methylene Blue (EMB), Bile Esculin slants, Triple Sugar Iron (TSI) (all from Difco), and Blood agar, were employed to characterize ANT 17 taxonomically. Components of non-commercial media are listed in Appendix B.2. These media were used to characterize antibiotic resistance, motility, salt tolerance, and metal resistance.

2.1.3. Media used to assess growth on petroleum-related compounds

Several methods were used to qualitatively assess growth of ANT 17 on various petroleum-related compounds, including liquid and solid media. In addition, growth was assessed when volatile compounds were supplied in the vapour phase. Appendix B.3 lists the components of the media.

Appendix E lists the chemicals used as carbon sources. Solid compounds (Section 2.6 and Appendix E.1) added to liquid cultures were prepared as concentrated stocks in N,N-dimethylformamide (DMF, Anachemia, Montreal, PQ) and added to the medium to achieve the desired final concentration (up to 5 mmol/L). Concentrated DMF stocks were stored in amber microfuge tubes to reduce photooxidation. Compounds were applied to solid media by spraying an ether solution of the compound onto mineral agar or BYP agar, or by suspending the compounds within agarose overlaid on mineral agar, or by suspending the particulate compound within mineral agar. For volatile compounds,

the compound was provided by placing solid crystals on the petri plate lid or by providing liquid compounds in a glass tube fixed to the petri plate lid. Appendix B.3 provides details of the procedures for both solid and vapour phase carbon utilization assessment.

When oils or refined products were supplied as the sole C and energy source, they were added directly to the sterile liquid culture medium after inoculation. A sterile glass pipette was used to add either 200 or 400 μL of oil per culture flask. The oils have been shown in previous experiments (J. Foght, personal communication) to be sterile. Some of the oil cultures were amended with N and P nutrients aseptically added from a sterile 50X stock solution (N,P; Appendix B.3) to the medium just prior to the addition of the oil.

For quantitative studies of growth of ANT 17 on various petroleum–related compounds, where growth was measured as protein concentration (Section 2.9), Mueller mineral salts medium (Mueller *et al.*, 1989, Appendix B.3) was utilized to avoid interference with the protein assay seen with mineral medium.

Semi-quantitative growth studies which included Tween® 80 (JT Baker, Phillipsburg, NJ) (final concentration 200 mg/L) utilized phenanthrene, fluoranthene, and pyrene stocks dissolved in acetone, and chrysene and anthracene dissolved in chloroform, to achieve a final suspension of 100 mg of PAH/L. The PAHs were added to sterile flasks and the solvent was allowed to evaporate (through a foam plug) prior to the addition of sterile Mueller mineral salts medium and inoculum. This method was used to assess growth when the PAH was supplied in the absence of solvent ("dry"), compared with conditions where the PAH stock was added to sterile medium directly without allowing evaporation of the solvent ("wet").

Quantitative analysis of growth on *n*-alkanes (*n*-C_5, *n*-C_6, *n*-C_{11}, *n*-C_{12}, *n*-C_{16}, *n*-C_{17}) and hexanoic acid involved the addition of these liquid compounds directly to sterile Mueller mineral salts medium. Hexadecanoic acid was dissolved in a concentrated stock of propyl alcohol prior to its addition. Controls to account for growth on the propyl alcohol solvent were included.

2.1.4. Buffers and solutions

Sterile 10 mM phosphate buffer (pH 7.2; Appendix B.4) was used to resuspend cells harvested from broth or plates, to facilitate replicate inoculation of media. For example, after a "seed" culture was grown in a rich medium such as TSB, the culture would then be harvested by centrifugation (6,000 x g for 10 min) and resuspended in an equal or lesser volume of this buffer, to be inoculated into a mineral culture medium. This method was used to assess growth on petroleum-related C sources because it avoided contamination of the mineral medium with nutrients from the rich broth culture. This buffer was also used for serial dilutions of cells for plating, and as a diluent for BCA assay samples.

For the analysis of growth at various pH values, QTSB was buffered to give an initial pH of 5.8 to 9.0. Appendix B.4 describes the buffers employed (Gerhardt *et al.*, 1994). One hundred and twenty five millilitre flasks containing 50 mL of medium were used. The initial pH of the culture medium was determined after autoclaving because the initial pH changed slightly due to CO_2 loss during heating. Both OD_{600} readings and protein concentrations were used to quantify growth in this medium.

2.2. Maintenance cultures

ANT 17 was preserved in 25% glycerol medium at –70°C (Sambrook *et al.*, 1989). To create a stock inoculum plate, ANT 17 was streaked from a glycerol stock to a phenanthrene overlay plate and a 1-methylnaphthalene vapour plate. This ensured that the PAH^+ phenotype of ANT 17 was maintained and that the inoculum for each experiment was as close to the original isolate as possible. Streaking to PCA assessed purity of the glycerol stock. All inoculum plates were incubated at 22° or 28°C in the dark, typically for 3 to 5 days, and used fresh.

2.3. Culture conditions

All incubations were carried out in the dark. Tube cultures were incubated on a tube roller (model TC-7, New Brunswick, NJ) set to a speed of 5 for tubes containing up to 10 mL of liquid medium (18 x 150 mm glass test tube capped with Kim-Kaps [Kimble Glass Co., distributed by Fisher]). Agar plates were sealed with Parafilm (American National

Can., IL) and plates containing a vapour phase carbon source were placed in a bag separate from other plates to prevent vapour cross-contamination. For non-radioactive experiments, shake flasks were sealed with foam plugs and capped with foil to allow gas exchange (except for early studies with Tween® 80 that utilized flasks sealed with screw caps lined with Teflon). Flasks used with volatile C sources were sealed with Neoprene stoppers (Kontes, NJ). Radioactive experiments in side arm flasks (Fedorak *et al.*, 1982) required rubber septa (Arthur H. Thomas Co. PA) that were suitable for sampling using a needle and syringe.

Flasks typically were incubated on a gyrotory shaker (New Brunswick, NJ) with a speed of 150 to 200 rpm. Incubation was carried out at 1°, 4°, 8°, 10°, 15°, 28°, 30°, 35° or 37°C in controlled environment chambers (± 2°C). The exception was the ambient incubator (ca. 22°C) that was not controlled.

2.4. Taxonomy

2.4.1. General taxonomic determinations

The Gram stain, wet mount, KOH test, catalase test, and oxidase test were conducted as described by Gerhardt *et al.* (1994).

2.4.2. API 20E system and API 20NE identification of non-enteric Gram-negative rods

These tests were carried out essentially as described by the manufacturer (bioMérieux Vitek Inc., MO).

2.4.3. Microscopy

Dilute crystal violet was used to aid in the visualization of ANT 17 cells during wet mount microscopic analyses. A few drops of crystal violet (Gram stain reagent; Gerhardt *et al.*, 1994) were diluted with sterile ddH_2O and placed near the edge of the wet mount and allowed to diffuse into the cell suspension. This stained the cells purple and improved contrast. The Acid Fast, Capsule, and Metachromatic Granule staining procedures (Norrell and Messley, 1997) are described in Appendix C. Bright-field

microscopy (Ernst Leitz GMBH Wetzlar, model 307-127.OC2, Germany) was used to observe all stained preparations.

2.4.4. Pigment analysis

To examine the nature of the yellow pigment produced by ANT 17, a procedure described by Pollock (1993) was followed. A few isolated colonies were removed from a PCA plate and placed into a 1.5-mL microfuge tube containing 500 µL methanol. This was incubated in a 60°C waterbath for 5 min to allow extraction of the pigment. The cells were then removed by centrifugation and the supernatant was scanned from 400 to 600 nm using a scanning spectrophotometer (Philips, model # PU 8740, England) to determine the characteristic absorption maxima.

2.4.5. Resistance to Ultraviolet (UV) radiation

ANT 17 was pre-grown in TSB, and the cell pellet was washed and resuspended in 10 mM phosphate buffer (pH 7.2) to dilute the cells and to remove nutrients that may absorb UVA radiation and interfere with the assay. The cell suspension was placed in an empty sterile plastic Petri plate and an unirradiated time zero sub-sample was plated onto PCA. With the plate lid off, the washed cell suspension was exposed to longwave UV for a total of 20 min, with sub-samples taken at intervals. A Chromato-Vue UV cabinet (model C5, Ultra-Violet Products, San Gabriel, CA) was utilized for the exposure, with a nominal longwave peak intensity at 365 nm of 2200 $\mu w/cm^2$ on the cabinet floor. The plates were incubated at 22°C for 5 days in the dark and scored for colony forming units. As a UV-resistant control, *Micrococcus radiodurans* NCIB 9279 was similarly assayed for a decrease in viability, as this organism is known for its UV and γ-radiation resistance.

2.4.6. Survival after cycles of freezing and thawing

The viability of *S. paucimobilis* strains ANT 17 and WW3 and *E. coli* were compared after cycles of freezing and thawing. Cultures were inoculated into 50 mL TSB and incubated at 22°C until turbid. One-millilitre portions of the TSB culture were transferred to sterile Nalgene (Nalge Nunc International, Milwaukeee, WI) cryovials.

Vials of each set were placed in an isopropanol bath and frozen for 24 h at –20°C. A vial was used for a time zero enumeration of the cells. The entire vial was quantitatively transferred to 9 mL of 10 mM phosphate buffer (pH 7.2, 10^{-1} dilution) and this tube was subsequently diluted serially and plated onto PCA in triplicate. Plates were then incubated at 22°C until counted. The remaining vials were treated with a cycle of freezing for 24 h, at which time the entire set of vials was transferred to 22°C in the dark for a thaw cycle of 24 h. Upon completion of the thaw cycle, the contents of one of the vials was diluted and plated as described for the time zero analysis. The remaining vials were then frozen again to initiate another cycle. This process was repeated for nine cycles in total.

2.5. Molecular studies

2.5.1. Plasmid DNA preparation

Plasmid DNA preparations were conducted essentially as described by Keiser (1984). Appendix D details the procedure and lists the components of the reagents used.

2.5.2. Chromosomal DNA preparation

Chromosomal DNA preparations were carried out as described by Marmur (1961), with several modifications. Appendix D details the procedure and lists the reagents used.

2.5.3. Gel electrophoresis

Gel electrophoresis was used to analyze plasmid and chromosomal DNA obtained by the methods described above. A 0.7% w/v agarose (UltraPure, Life Technologies Inc., MD) gel prepared in TEA (Appendix D) was poured into a mini-gel apparatus (BioRad, Richmond, CA). TEA buffer was also used as the running buffer. Samples were loaded with 5X loading dye (Sambrook *et al.*, 1989) and compared to a λHindIII digest molecular weight marker (Boehringer Mannheim, Canada). Gels were run at 80 V for 1.5 h (VWR Scientific #105 power-pack). Appendix D lists the composition of the buffer, gel and loading dye. The gel was transferred to a dilute ethidium bromide solution for staining (30 min) followed by a 10 min destaining period in ddH_2O. Bands

on the gels were visualized under UV light and recorded using a Mitsubishi video copy processor and a digital camera (The Imager, model 4912-2010/0000).

2.5.4. CHEF – PFGE

The protocol followed for the preparation of DNA for CHEF analysis was as described by the manufacturer of the apparatus (BioRad) with some modifications, as listed in Appendix D. The specifics of the CHEF system were: CHEF-DrII system (PFGE, BioRad), model 200/2.0 power supply (200 V/2.0 amps) and a pulsewave 760 switcher (760 V maximum).

2.6. Chemicals

All chemicals, suppliers, and purities (where documented) are listed in Appendix E.1.

2.7. Radiochemicals

Mineralization studies utilized the following radiochemicals: $H^{14}CO_3$ (bicarbonate, Amersham, Batch 188), [3-^{14}C]-fluoranthene (Sigma, lot # 114H0196, 96% pure), [U-^{14}C]-glucose (New England Nuclear, Boston, MA, lot # 641-198), *n*-[1-^{14}C]-hexadecane (Amersham, Batch 31, 97% pure), [1-^{14}C]-palmitic acid (New England Nuclear, lot # 805.015), [9-^{14}C]-phenanthrene (Amersham, lot # NSB/1/175, 98% pure), and [4,5,9,10]-^{14}C-pyrene (Sigma, lot # 18H9602, >99% pure).

2.8. Crude oils and refined oil products

2.8.1. Extraction of residual oil

Cultures and sterile controls containing crude oils or refined products were acidified with 2 mL 4N H_2SO_4 to pH <2. A stock solution containing squalane and *o*-terphenyl (Appendix E.2) was added as the surrogate extraction standard, using a glass volumetric pipette. The flask contents were transferred to a 500-mL separatory funnel and extracted with multiple potions of dichloromethane (DCM; HPLC-grade). At a minimum, three 20-mL portions were used and the extraction was not considered complete until the organic phase was colourless. The solvent phase was collected and dried by passage

through anhydrous Na_2SO_4. The pooled solvent extract was concentrated to approximately 1 mL by rotary evaporation (Roto-vap; Brinkmann, ON) in a water bath at 50°C and quantitatively transferred to a glass dram vial with a Teflon liner. Further concentration under a stream of N_2 gas was performed, if required, to obtain a volume of approximately 2 mL. This sample was then split into two equal portions and stored at 22°C in the dark in glass Dram vials with Teflon liners until fractionated (Section 2.8.2). This allowed one half of the sample to be fractionated while the other sample was archived as a reserve.

2.8.2. Fractionation of extracted oil

Fractionation of extracted oil was conducted by silica gel liquid column chromatography, essentially as described by Fedorak and Westlake (1981), but omitting the activated copper. Silica gel lot # 07201MQ was used (Aldrich, WI). The column was developed in 10 mL *n*-pentane, 20 mL 20% DCM in *n*-pentane, and 25 mL 50% DCM in *n*-pentane. These elution volumes were modified so that 10 to 12 mL were collected as the void volume, 20 mL collected as the saturated fraction and the remainder (25 mL) collected as the aromatic fraction. The aliphatic and aromatic fractions were concentrated by rotary evaporation and transferred to 2-mL glass autosampler vials with Teflon-lined septa (Hewlett Packard, PA).

2.8.3. GC-FID analysis of extracted residual crude oil

Fractionated oil samples were analyzed on a Hewlett-Packard 5890 GC with FID detection and a DB5 (J&W Scientific, CA) 30 m x 0.25 mm ID, 0.25 μm fused silica capillary column. Samples of 2 μL were injected either manually, using a 10-μL glass syringe (Hamilton Co., NV) or via an autosampler device (Hewlett-Packard 7673). The temperature program was set at 90°C for 2 min followed by an increase of 4°C/min to a final temperature of 250°C which was held for 18 min, giving a total run time of 1 h for each fraction analyzed.

2.9. Protein determination

The bicinchoninic acid (BCA) protein assay (Pierce Chemical Co., Rockford, IL) was used for all determinations of culture protein concentration. The test tube protocol (using the enhanced protocol for incubation times) was followed as described in the manufacturer's instructions. One-millilitre samples of liquid cultures were clarified in a microfuge for 10 min at maximum speed (13, 000 x g). The supernatant was removed by aspiration. For cultures grown in TSB or QTSB, the cell pellet was washed in 1 mL of 10 mM phosphate buffer (pH 7.2), pelleted again and the supernatant aspirated. Cell pellets were then frozen at –20°C until assayed.

Thawed cell pellets were resuspended in 0.1M NaOH and lysed by incubation in a 60°C water bath for 30 min. The lysed samples were then transferred to 13 x 100 mm test tubes, 2 mL of working reagent was added (Appendix F), mixed by vortex, and incubated again at 60°C for 30 min. Spectrophotometric readings were obtained at A_{562} with the spectrophotometer blanked with ddH_2O. A blank is included in the standards used for the generation of a calibrations curve (Appendix F) and is subtracted from all sample and standard readings. Bovine serum albumin (BSA; albumin standard ampules included with the assay kit) was used as the standard protein. The BCA assay is suitable for protein concentrations ranging from 5 to 250 µg/mL BSA protein equivalents. Samples that were outside this range were diluted with ddH_2O prior to reading. For especially turbid cultures, 0.1-mL samples were taken and brought to 1 mL using 0.9 mL 10 mM phosphate buffer and processed as a 1 mL sample. The BCA assay was used as opposed to more conventional protein assays, due to its compatibility with a wide variety of chemicals and reagents. Specifically, the BCA assay is accurate in the presence of Tween 80 (up to 1%), DMF (up to 10%), and NaOH (up to 1 M).

2.10. pH determination

All pH readings were obtained using a Fisher model 620 pH meter equipped with a pH probe (Accu-pH, Fisher) calibrated at room temperature with a standard pH 7.0 buffer (VWR Scientific).

2.11. Mineralization Studies

2.11.1. Stock solutions of radiolabelled compounds

Concentrated stock solutions of radiolabelled compounds (listed in Section 2.7) were prepared by combining a suitable quantity of radiolabelled compound diluted with a known amount of non-radiolabelled compound. This was conducted so that a desired concentration of compound (up to 2.5 mmol/L) would be achieved with a suitable amount of radioactivity (from 50 000 to 500 000 dpm total per culture). Stock solutions were prepared in DMF, except for glucose (7:1 ethanol:water). The stock solutions were then filter sterilized through solvent-stable Millex 0.22-µm FG syringe tip filters (Millipore) and stored at –20°C. The actual dpm/mL in each stock solution was quantified for calculation purposes.

2.11.2. Culturing techniques

Cultures with radioactive compounds were prepared in sealed flasks as described in Section 2.3 and inoculated prior to the addition of the C source and subsequent sealing of the flasks. Time zero was recorded as the time at which the stock solution was added. Fifty millilitres of either Mineral medium (Appendix B.3) or modified Mineral medium were utilized in the 125-mL side-arm flasks. The modified Mineral medium was buffered to pH 6.4 (Appendix B.4) and included 0.01% yeast extract. This was done to optimize the conditions for ANT 17 growth (by buffering) and to parallel the medium content of overlay plates (containing 0.01% yeast extract, Appendix B.3).

2.11.3. Analysis for $^{14}CO_2$ and liquid scintillation

The procedure and apparatus utilized for analyses of $^{14}CO_2$ were conducted essentially as described by Fedorak *et al.* (1982). Modifications were made to account for the reduced flask size and volume of medium. At each sampling time, 2 mL of headspace and 1 mL of the liquid medium were removed through the sampling port (sterilized by an alcohol pad) by a sterile 25G 5/8" needle and 5-mL Luer-Lok syringe (Becton Dickinson, Franklin Lanes, NJ). The sample was injected into an evacuated 30-mL serum bottle containing 2 mL 4 N H_2SO_4. To prevent a vacuum from developing within the culture flask with repeated sampling, 3 mL of air was injected into the culture

flask through a 0.22 μm vertical syringe tip filter (μStar® LB™ #8110, Costar). Flushing of the acidified sample within the serum bottle was conducted with an apparatus described by Fedorak *et al.* (1982) except that two trapping vials were used instead of three vials. Scintillation vials (20 mL, Fisher) were filled with 10 mL Aqueous Counting Scintillant (ACS fluor, Amersham, Oakville, ON) and 1 mL Carbosorb-E (Packard, Meridian, CT). The flushing and trapping apparatuses employed were greater than 96% efficient at trapping $^{14}CO_2$ as determined with a bicarbonate standard. Radioactivity was determined after 10 min of dark adaptation in a Beckman LS 3801 scintillation counter. Samples were counted for 2 minutes and counts per minute (cpm) were converted to disintegrations per minute (dpm) by the H# calculation program (Beckman). Dpm values were utilized in all calculations.

2.11.4. Distribution of radiolabel

Radiolabel distribution analysis (Section 3.8.2) was conducted after the final sampling and analysis for $^{14}CO_2$. Five millilitres of the remaining culture or sterile control were counted directly for radioactivity. Ten millilitres of each flask were filtered through loosely packed glass wool, with two 5-mL washes of 10 mM phosphate buffer. The glass wool was counted directly, as this contained residual insoluble precipitate from the radiolabelled carbon source. The filtrate was centrifuged to remove cellular material. The pelleted cells were lysed in 5 mL 0.1M NaOH in a 60°C waterbath for 30 min. The lysate was then neutralized with 2 mL 4 N H_2SO_4 to prevent quenching during scintillation counting, and the entire 7-mL sample was counted. The 20 mL supernatant after centrifugation was acidified with 2 mL 4 N H_2SO_4 and subjected to two 5-mL DCM extractions. The 10 mL solvent phase was counted as DCM extractable metabolites. The remaining aqueous solution after the DCM extractions was also counted (5-mL aliquot). The emptied flasks were rinsed with DCM to account for radiolabel adhering to the glass. This procedure was utilized for cultures containing radiolabelled phenanthrene, hexadecane, pyrene, and fluoranthene.

The radiolabel distribution protocol for radiolabelled glucose cultures was modified by omitting the glass wool filtration (as there was no insoluble precipitate present in this

culture), and the DCM extraction. Radiolabel distribution for cultures containing palmitic acid were modified by omitting the DCM extraction.

3. RESULTS AND DISCUSSION

3.1. Characterization of ANT 17

This portion of the thesis research was conducted to characterize ANT 17 taxonomically as well providing background data for preliminary molecular studies.

3.1.1. Taxonomic characterization

3.1.1.1. Cell and colony morphology

ANT 17 is a Gram-negative rod. Chains of two to three cells were observed in a 1-day-old NBG culture consisting of cells of approximately 1 μm x 0.5 μm. Growth in shaking broth cultures was often observed as a turbid culture with an aggregated mass of cells on the bottom of the culture tube or flask. On PCA plates, ANT 17 grew to form bright yellow colonies 1.5 to 3 mm in diameter in 3 days at 30°C. The colonies were convex, circular, and smooth with an entire edge. Yabuuchi *et al.* (1990) noted that organisms belonging to the genus *Sphingomonas* were Gram-negative rods with yellow pigmentation.

3.1.1.2. Growth on selective and differential media

On blood agar, ANT 17 grew as yellow colonies, associated with minimal clearing as well as a blackening of the blood in the surrounding medium (α-haemolysis). No haemolysis on sheep blood agar was observed with the *Sphingomonas* spp. of the Yabuuchi *et al.* (1990) study. However, Minnick and Scherer (1993) have isolated, cloned, and sequenced a haemolysin-encoding gene from *S. paucimobilis* isolated from tap water. No growth was observed on KF agar (used to differentiate fecal streptococci on the basis of sodium azide inhibition of respiring organisms, growth by fermentation of maltose and lactose). No growth was seen on MacConkey's agar indicating that ANT 17 does not utilize lactose or is inhibited by bile salts or crystal violet (red or pink colonies on this medium normally indicate a lactose fermenter). Yabuuchi *et al.* (1990) did not observe growth of any *Sphingomonas* isolates on MacConkey's agar. Reddish – pink colonies were observed on EMB agar, confirming that ANT 17 is a lactose non-fermenter. ANT 17 grew on and blackened a bile esculin slant, indicating that it could

tolerate 4% bile and could hydrolyze esculin to glucose and esculetin (esculetin combines with ferric salts in the medium to produce the black colour). Growth in bile esculin has been recorded for all *Sphingomonas* spp. that have been identified (Yabuuchi *et* al., 1990). A TSI plate inoculated with ANT 17 showed yellow growth but no blackening was observed. This medium is used to differentiate Gram-negative bacilli based on fermentation of dextrose, sucrose, lactose, and on H_2S production.

3.1.1.3. Motility assessment

Dilute crystal violet was used to aid visualization of ANT 17 cells in a wet mount prepared from a 1-day-old NBG culture. Because no motile cells were observed, Bacto Motility Test medium was used to aid in the determination of motility ANT 17. A stab of cells was inoculated into the sloppy agar and growth was observed along the "stabline" as well as along the top of the agar surface; no growth was observed beyond the "stabline", again indicating a lack of motility. However, during a separate microscopic examination of an ANT 17 liquid culture grown on a hydrocarbon, motile cells were observed. Therefore, it appears that the motility of ANT 17 is variable and dependent upon specific cultural conditions, but the factors responsible for determining motility were not examined. Motility was observed in 15 of the 16 *Sphingomonas* isolates analyzed by Yabuuchi *et al.* (1990).

3.1.1.4. Cell staining

In addition to the Gram stain, several other staining methods were applied to a 1-day-old TSB culture. ANT 17 was negative for the Acid Fast stain as well as for the capsule stain. Metachromatic granule (polyphosphate inclusion) staining revealed that ANT 17 contained one to two round bodies near the ends of the cells that stained dark blue, indicating that ANT 17 contained polyphosphate stored within inclusion bodies.

3.1.1.5. API 20E and API 20NE identification tests and other biochemical tests

The API 20E identification test indicated that ANT 17 was a pseudomonad. The API 20NE identification test was inconclusive; however it indicated that ANT 17 was similar to either *S. paucimobilis* or *Sphingomonas multivorum*. ANT 17 was also positive

for KOH, confirming its Gram negative nature. ANT 17 was also positive for catalase, confirming its aerobic nature. The 16 *Sphingomonas* isolates described Yabuuchi *et al.* (1990) were all strict aerobes and catalase positive.

3.1.1.6. Pigment analysis

The yellow pigment produced by ANT 17 was extracted and analyzed (Pollock, 1993). The scan revealed a major peak at 448 nm, a secondary peak at 476 nm and a shoulder at 424 nm (Figure 3.1). These results closely matched the published values for the carotenoid pigment nostoxanthin (Jenkins *et al.*, 1979) and the observed absorption pattern of *S. paucimobilis* WW3 (Figure 3.1; see Section 3.1.2) with the major peak at 449 to 451 nm, the secondary peak at 475 to 478 nm and the inflection from 415 to 430 nm. This result is characteristic of a *Sphingomonas* species (Pollock, 1993).

3.1.1.7. Antibiotic resistance assessment

ANT 17 was tested for resistance to several antibiotics as described in Appendix B.2. Agar plates were utilized for this assessment and growth or lack of growth (within a measured radius from the edge of the antibiotic disc) in the presence of each antibiotic disc demonstrated resistance or sensitivity. ANT 17 was sensitive to tetracycline (20 mm clearing zone), novobiocin (15 mm clearing zone), chloramphenicol (13 mm clearing zone with an additional 7 mm zone of diffuse growth) and resistant to streptomycin (clearing zone of only 1 mm). Penicillin G was included as a control and, as expected, ANT 17 was resistant to this antibiotic, being a Gram-negative organism. ANT 17 was also sensitive to rifampicin and kanamycin (dissolved within the agar medium; Appendix B.2). Known resistant organisms were not included in this analysis for comparison. The 16 *Sphingomonas* isolates studied by Yabuuchi *et al.* (1990) were chloramphenicol and ampicillin sensitive and streptomycin resistant.

The sensitivity of ANT 17 to the antibiotics tested is not surprising as this organism was isolated from an environment where exposure to antibiotics would not be expected, thus limiting the chances for resistance to develop. The sensitivity of ANT 17 to many antibiotics could allow the use of this organism in a commercial or industrial setting with the ability to treat workers in the event of an infection. An organism resistant to many

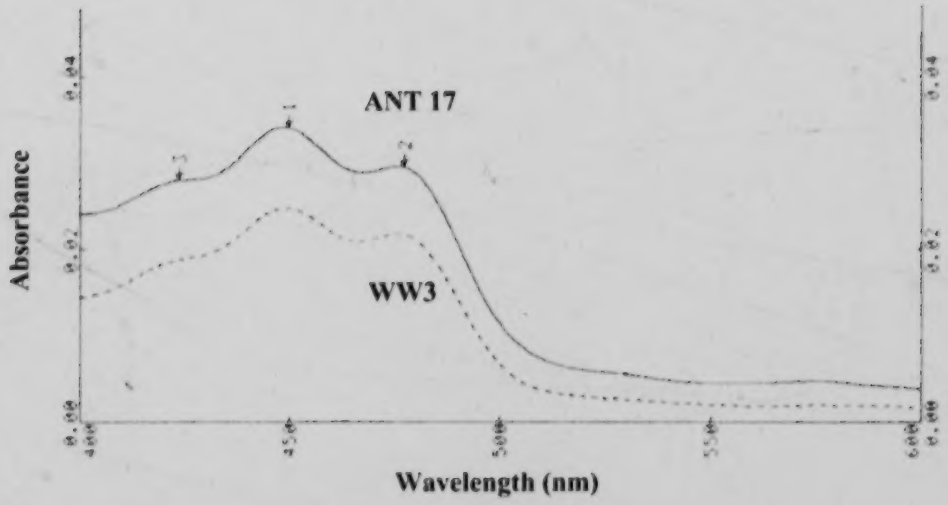

Figure 3.1. Absorbance spectrum of the methanol-extractable pigment from *Sphingomonas* spp. ANT 17 and WW3. Major peaks occur at 448 nm (1) and 476 nm (2) and a shoulder at 424 nm (3). ANT 17 (—) and WW3 (--).

types of antibiotics is less attractive for commercial applications due to the increased risk to workers

3.1.1.8. FAME analysis

These results were provided by Dr. J. Aislabie, LandCare Research, New Zealand. The results of this analysis indicated that ANT 17 is not identical to any genus in the environmental database of LandCare Research, New Zealand. Table 3.1 compares the results from ANT 17 to those of *Sphingomonas* species reported by Yabuuchi *et al.* (1990), Pollock (1993) and Stead (1992).

The data in Table 3.1 shows that ANT 17 is quite different in fatty acid composition from other *Sphingomonas* spp. The quantity of 2-hydroxymyristic acid in ANT 17 is much less than in the clinical isolates analyzed by Yabuuchi *et al.* (1990) upon classification of this genus. The presence of 2-hydroxymyristc acid (18 to 31%) and *cis*-octadecanoic acid (44 to 63%) was described by Yabuuchi *et al.* (1990) as distinguishing quantities of fatty acids in the profiles of *Sphingomonas* spp. (discussed in Section 1.7). The environmental isolates analyzed by Stead (1992) were closer to ANT 17 in the quantity of 2-hydroxymyristic acid. Compared to the other mesophilic *Sphingomonas* isolates described in Table 3.1, ANT 17 has a noticeable reduction in the quantity of saturated fatty acids in favour of an increase in unsaturated fatty acids. The quantity of hexadecanoic acid is greatly reduced compared to the increase in *cis*-unsaturations. In addition, ANT 17 does not contain *cis*-11 octadecanoic acid, but instead possesses a large and comparable quantity of the *trans*-monounsaturations of octadecanoic acid.

The functional differences for ANT 17 to have such a distinct profile most likely stem from cold tolerance. As described in Section 1.4, decreasing the acyl chain length and increasing the number of unsaturations are part of the survival strategies employed by microorganisms to maintain membrane fluidity under cold conditions. ANT 17 appears to employ both strategies. The number of unsaturations is greater than in the temperate isolates. In addition, by comparing the quantity of the octadecanoic acids to the hexadecanoic acids, one can see a reduction in the longer chain fatty acids in favour of an increase in hexadecanoic acids.

Table 3.1. Comparison of ANT 17 FAME analysis with published values for other *Sphingomonas* spp.

	Fatty acid (Percentage in profile)[a]								
Strain	**14:0**	**14:0 2-OH**	**15:0**	**16:0**	**16:0 iso 3-OH[f]**	**16:1 ω5c[f]**	**16:1 *cis*-9**	**16:1 C**	**16:1ω7c or 15 iso 3-OH[f]**
S. paucimobilis[b]	1.1	24.3	0	10.7	-[e]	-	3.0	-	-
	(0.4)	(5.2)	0	(1.1)	-	-	(1.5)	-	-
S. paucimobilis[c]	0.6	7.7	0	14.0	-	-	8.2	1.0	-
	(0.2)	(1.3)	0	(1.8)	-	-	(1.3)	(0.1)	-
tentative	0.1	11.6	0.1	18.7	-	-	2.1	0.6	-
Sphingomonas spp.[d]	(0.4)	(3.2)	(0.4)	(4.7)	-	-	(1.6)	(0.8)	-
ANT 17	0.90	5.43	-	4.0	2.44	1.77	-	-	31.63

	Fatty acid (Percentage in profile)								
Strain	**17:0**	**17:1 C**	**17:1 ω6c[f]**	**17:1 ω7c[f]**	**18:0**	**18:1 B**	**18:1 *cis*-11**	**18:1 ω5c[f]**	**18:1ω7t or ω9t or ω12t[f]**
S. paucimobilis[b]	0	1.0	-	-	0	0	58.0	-	-
	0	(0.1)	-	-	0	0	(3.6)	-	-
S. paucimobilis[c]	0	1.0	-	-	0.8	1.1	65.2	-	-
	0	(0.7)	-	-	(0.2)	(0.2)	(1.0)	-	-
tentative	0	1.9	-	-	0	0	64.0	-	-
Sphingomonas spp.[d]	0	(2.3)	-	-	0	0	(2.1)	-	-
ANT 17	-	-	1.21	1.07	-	-	-	1.20	49.36

[a] 14:0, tetradecanoic (myristic) acid; 14:0 2-OH, 2-hydroxytetradecanoic acid; 15:0, pentadecanoic acid; 16:0, hexadecanoic (palmitic) acid; 16:1 *cis*-9, *cis*-9-hexadecaonic (palmitoleic) acid; 16:1 C, hexadecanoic acid isomer C; 17:0, heptadecanoic (margaric) acid; 17:1 C, heptadecanoic acid isomer C; 18:0, octadecanoic (stearic) acid; 18:1 B, octadecanoic acid isomer B; and 18:1 *cis*-11, *cis*-11-octadecanoic (*cis*-vaccenic) acid.

[b] Means (and standard deviations) for seven different strains of *S. paucimobilis* (Yabuuchi *et al.*, 1990).

[c] Means (and standard deviations) for four different strains of *S. paucimobilis* (Stead, 1992).

[d] Means (and standard deviations) for seven different tentative strains of *Sphingomonas* sp. (Pollock, 1993).

[e] -, not detected

[f] Positions of unsaturations are indicated as either in the *cis* (c) or *trans* (t) configuration, numbered from the "omega" (ω) end of the fatty acid; methyl braching indicated as "iso"; unresolved peaks from,the analysis listed as more than one possible fatty acid component for the percentage given.

The role of sphingoglycolipids as paralleling the role of LPS was discussed in Section 1.7.1. In a study by Wiese *et al.* (1996), it was noted that at lower temperatures the asymmetric bilayer containing sphingoglycolipids was more fluid (less ordered) than the asymmetric bilayer containing LPS. At 37°C the physical state of both asymmetric bilayers were identical. Perhaps the presence of sphingolipids in the outer membrane would enhance the ability of *Sphingomonas* spp. to grow at lower temperatures compared to other Gram-negative bacteria containing only LPS in the outer membrane. Further research is required in this area to determine the physical and biochemical consequences of the presence of these unique outer membrane constituents at low temperatures. Although the sphingoglycolipids of ANT 17 were not investigated in this study, it would be interesting to determine the presence and role of these outer membrane components, with respect to ANT 17 growth, in conjunction with the biodegradation studies described in the following sections of this thesis.

3.1.1.9. 16S rRNA analysis

Dr. J. Aislabie also has provided the results of the 16S rRNA analysis of ANT 17. These results have indicated that ANT 17 is most similar to *S. paucimobilis* (Aislabie *et al.*, 1999).

3.1.1.10. Summary of taxonomic characterization

From taxonomic studies conducted with ANT 17, it was identified as *S. paucimobilis*. Given that the Latin name *paucimobilis* means "few motile" and that motility of ANT 17 is variable, this classification would seem appropriate.

3.1.2. Comparison with *Sphingomonas* sp. WW3

A *Pseudomonas (Sphingomonas) paucimobilis* isolate WW3 (Weissenfels *et al.*, 1990) was used for comparative purposes in various tests performed with ANT 17. WW3 was originally isolated from a soil enrichment culture. WW3 grows on and clears phenanthrene as well as growing on 1-methylnaphthalene, like ANT 17. It was therefore suitable for comparison with ANT 17.

A pigment scan conducted with WW3 is shown in Figure 3.1, illustrating the similarities to the ANT 17 results. An API 20NE test conducted with WW3 showed a good match to *S. paucimobilis*, as expected as this isolate was originally identified in this manner (Weissenfels *et al.*, 1990). The only difference between ANT 17 and WW3 in this test was the ability of WW3 to assimilate maltose, whereas ANT 17 was unable to do so. WW3 was also used in other growth studies for comparison to ANT 17 (Sections 3.2 to 3.4 and 3.7).

3.1.3. Molecular studies

3.1.3.1. Phenotypic stability

An attempt was made to "cure" ANT 17 of its ability to degrade and grow on phenanthrene and transform dibenzothiophene. These two compounds were used for this test based on the results presented in Section 3.6. ANT 17 was grown in TSB medium at 28°C on a tube roller in the dark and transferred daily to fresh medium for 2 weeks. At the end of the 2-week period, the culture was plated onto PCA. Fifty colonies were randomly picked and streaked to both a phenanthrene overlay plate and a BYP plate. All 50 colonies maintained the ability to grow on and clear phenanthrene. In addition, after spraying the parallel BYP plate with dibenzothiophene, all 50 colonies transformed this heterocycle to an orange product, presumably the ring cleavage product (Foght and Westlake, 1988). Since ANT 17 did not lose the ability to utilize phenanthrene or transform dibenzothiophene even after repeated transfers in rich medium, its phenotype was determined to be stable.

3.1.3.2. Studies to determine the presence or absence of plasmid(s)

Small-scale genomic DNA isolations (Marmur, 1961) as well as plasmid preparations (Kieser, 1984) were conducted to determine if ANT 17 contained plasmids. Such plasmids could possess catabolic genes for biodegradation of compounds found within crude oil. Plasmids are known to contain genes for the degradation of a wide variety of compounds and have been extensively isolated and reported (Leahy and Colwell, 1990). No plasmids were observed in ANT 17 in these analyses, although parallel positive control cultures yielded plasmids: *Pseudomonas putida* NCIB 9816 (NAH2, 87 kb;

NAH3 64 kb), *Pseudomonas fluorescens* LP6a (pLP6a, 63 kb) and *P. putida* PpG (NAH7, 87 kb).

It has been noted in several studies with *Sphingomonas* species that some strains contain large (catabolic) plasmids that are easily sheared during conventional DNA and plasmid preparations (Kim *et al.*, 1996; Feng *et al.*, 1997). These researchers utilized CHEF gel analysis, a form of PFGE, to assess the presence of large plasmids. Some strains contained plasmids of 130 to 270 kb and one notable isolate contained a plasmid of 800 kb (Kim *et al.*, 1996). Plasmids this large can be readily observed using PFGE because the cells are lysed within the agarose itself, preventing breakage of large, fragile plasmids. In addition, PFGE allows one to observe and determine the size of these plasmids, compared to size standards. The separation of supercoiled DNA (80 to 300 kbp) was found to be independent of the switch times used in PFGE (Wang and Lai, 1995). All supercoiled DNA was found to migrate to approximately the same position in the gel, regardless of the switch time used. The pulsing action of the current causes the DNA to reorient itself throughout electrophoresis and thereby prevents reptation, which normally causes all large molecules of DNA over a certain length to migrate to the same position of the gel (the exclusion limit) and thus appear to be of the same size.

For these reasons, the determination of the plasmid content of ANT 17 included CHEF gel analysis in case ANT 17 contained very large plasmids. However, no plasmids were observed. *Streptomyces clavuligerus* NRRL 3585 was included as a positive control. This organism contains two large linear plasmids (pSCL2, 120 kb; pSCL3 430 kb, Netolitsky, 1995) both of which were observed in parallel tests.

The fact that ANT 17 did not appear to contain any (catabolic) plasmids, supports the observation that ANT 17 appears phenotypically stable (Section 3.1.3.1). If ANT 17 does not contain plasmids, gene(s) encoding catabolic enzymes necessary for biodegradation must be chromosomal.

3.2. Tolerance of ANT 17 to environmental stresses

The purpose of this portion of the research was to assess whether or not ANT 17 possessed the ability to tolerate and possibly survive and proliferate under harsh environmental conditions simulated within the laboratory.

3.2.1. Salt tolerance

Growth of ANT 17 was tested in TSB medium with NaCl concentrations ranging from 0% to 8% w/v NaCl. OD_{600} readings were used to quantify growth, compared to uninoculated medium. The test was repeated twice and readings were conducted at 17 or 24 h after incubation in the dark at 22°C on a tube roller. Table 3.2 summarizes these results. ANT 17 grew well in 0% to 2.5% NaCl with 5% as the upper limit for growth. Salt tolerance at concentrations up to 2% NaCl indicate a halotolerant or facultative halophile (Tortora *et al.*, 1992). Ten of the 16 isolates studied by Yabuuchi *et al.* (1990) were able to grow in 3% NaCl.

Although the conductivity data of the isolation site of ANT 17 indicate that the soil was of low salinity (Appendix A.3.3), the soils of the Antarctic can become extremely saline. This is due to the low water content in these soils, which does not remove various organic and inorganic precipitates, as well as other contributing factors described in Section 1.6.1. The halotolerance of ANT 17 probably contributes to its survival under saline conditions when they exist.

3.2.2. UV resistance

The goal of this portion of research was to determine if ANT 17 exhibited any differences in viability when exposed to UV. UVA radiation (320 to 400 nm) readily reaches the Earth's surface. Antarctica is exposed seasonally to high levels of UVB (290 to 320 nm) radiation due to the reduction in ozone over this region (Vincent, 1988) as UVB is normally absorbed by ozone. UVC (<290 nm) radiation is absorbed by oxygen and does not reach the surface of the Earth, and is not as destructive as UVB radiation. The damaging effects of UV and near UV radiation have been discussed by Krinsky (1976) and summarized in Section 1.4.

Table 3.2. Increase in culture turbidity of ANT 17 incubated for 17 and 24 h at 22°C in TSB with 0 to 8% w/v NaCl.

% NaCl	17 h	24 h
0.0	1.59[a]	2.05
0.1	1.57	-
0.25	1.70	-
0.5	1.45	-
1.0	1.16	1.03
1.5	1.03	-
2.0	0.71	-
2.5	-[b]	0.29
5.0	0.13	0.01
6.0	-	0.00
7.0	-	0.00
8.0	-	0.00

[a] value represents net increase above initial inoculum turbidity
[b] not tested

This study focused on UVA radiation as described in Section 2.4.5. The CFU/mL of ANT 17 culture and the UV-resistant positive control (*M. radiodurans* NCIB 9279) surviving after up to 20 min exposure to longwave UV radiation (nominal cabinet peak intensity at 365 nm) are shown in Table 3.3. ANT 17 is resistant to UVA radiation, as no dramatic decrease in viable counts was observed when compared to *M. radiodurans*.

In a similar study, the survival of ANT 17 exposed to UV radiation in a biohazard hood was compared to *M. radiodurans*. The UV sources in Biohazard hoods are designed to kill microorganisms with more intense and shorter wavelength UV radiation. The UV cabinet of the previous study was older and may not have been at the specified radiation levels. ANT 17 did not survive the first 5 min, whereas *M. radiodurans* survived a 10 min exposure with a slight decrease in CFU/mL, but did not survive a 20 min exposure.

ANT 17 does seem to be tolerant of brief UV exposure, which may permit survival of cells near the soil surface in Antarctica. It is likely that the mechanism of resistance or tolerance to UV radiation stems from its carotenoid pigment, nostoxanthin. The protective effects of carotenoid pigments has been well documented (Krinsky, 1976) and is described in Section 1.4. *E. coli* transformed with plasmids containing carotenogenic genes for the production of various carotenoid pigments conferred UVB resistance (Sandmann *et al.*, 1998).

Further studies with more defined visible light and UV radiation sources could be conducted to determine the range and extent of ANT 17 resistance to radiation. In particular, a UV sensitive control (such as *E. coli*) would need to be included in these studies. It would also be interesting to determine the extent of this resistance due to the presence of nostoxanthin. If non-pigmented mutants of ANT 17 could be generated, these could be run in parallel to wild-type ANT 17 in order to determine the full protective capacity of this pigment.

3.2.3. Metal resistance

Many sites in the environment are polluted with more than one type of contaminant. This is especially true in areas of intense industrial processes, and petroleum-contaminated sites may be also contaminated with heavy metals and other industrial

Table 3.3. Survival of ANT 17 and *M. radiodurans*, measured as viable numbers (CFU/mL) after exposure to UVA radiation for various times.

Duration of exposure (min)	ANT 17	*M. radiodurans*
0	4.77×10^9	3.48×10^8
0.5	3.47×10^9	-[a]
1	4.05×10^9	3.06×10^8
5	1.23×10^9	1.95×10^8
10	3.46×10^9	2.10×10^8
15	2.39×10^9	1.93×10^8
17.5	2.95×10^9	-
20	2.77×10^9	2.03×10^8

[a]- no sample was taken

pollutants. It is therefore important to know if the organisms intended to carry out biodegradation of the desired chemical pollutant(s) are able to survive in regions with heavy metal contamination if so impacted.

Tolerance to Cu^{2+} and Hg^{2+}, provided as $CuSO_4 \cdot 5H_2O$ and $HgCl_2$, was tested using an agar dilution method (Nieto *et al.*, 1989, Section 2.1.2 and Appendix B.2). Twenty microlitres of an ANT 17 suspension was spotted onto each metal-containing plate as well as a control plate lacking metal. Plates were scored for growth after 4 days incubated at 22°C in the dark. ANT 17 grew well in Cu^{2+} concentrations up to 2.5 mM and weakly up to 5 mM. It also grew in the presence of Hg^{2+} up to 0.1 mM. According to the definitions of Nieto *et al.* (1989), an organism is considered copper and mercury tolerant if growth occurs in concentrations of at least 1 mM and 0.1 mM, respectively. ANT 17 is therefore tolerant of both metals by this classification.

Since ANT 17 is tolerant of these metals, it is likely that there are more metals that ANT 17 may tolerate. In the study of metal resistances of 250 moderately halophilic eubacteria by Nieto *et al.* (1989), many of these isolates were multiply metal resistant. More metal resistances of ANT 17 could be assessed, particularly if this organism were to ever be used as an inoculum for a petroleum contaminated site containing heavy metals. In previous years, many refined fuels were leaded. Older yet still persistent spill sites may contain high amounts of organic lead additives. Lead resistance would be essential to bioremediate these sites.

3.2.4. Survival after freeze-thaw cycles

This portion of research was conducted to determine the survivability of ANT 17 under cycles of freezing and thawing, as Antarctic soils are subject to rapid freeze-thaw cycles as described in Section 1.6.1. *S. paucimobilis* WW3 and *E. coli* were also tested to determine if the survivability of ANT 17 was unique to an Antarctic microorganism or unique to *Sphingomonas* spp. Figure 3.2 illustrates the results after nine freeze-thaw cycles conducted as described in Section 2.4.6. Cell enumeration was obtained in triplicate from plate counts after a 24-h freeze and 24-h thaw cycle. A time zero sample was included (cycle 0). Triplicate vials were plated (in triplicate) during cycle 8 for *E. coli* and cycle 9 for ANT 17 and WW3 to determine replication of the vial contents.

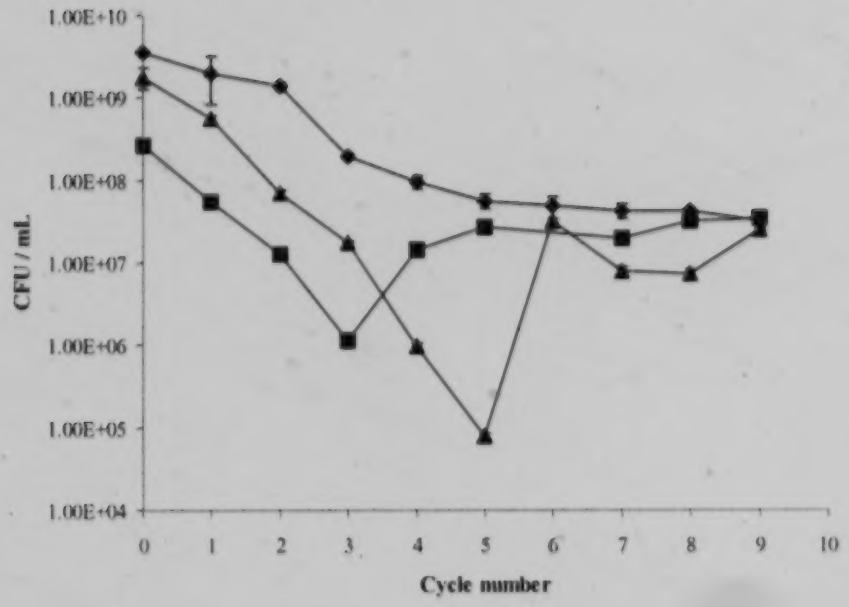

Figure 3.2. Survival of ANT 17 (▲), WW3 (■) and *E. coli* (◆) after cycles of freezing (24 h) and thawing (24 h), measured as CFU/mL. Each point represents the average of triplicate plate counts from a replicate cryo-vial; error bars (when visible) represent one standard deviation.

The viable counts were all within the same order of magnitude and therefore the replicate vials were representative.

After the first cycle, the reduction in cell number was 56%, 31% and 20% for *E. coli*, ANT 17 and WW3 respectively. After the dramatic reduction after the first cycle the viable numbers continued to decrease with each cycle but less dramatically. The viable cell number leveled out to approximately 4×10^7 CFU/mL for *E. coli* after three cycles (1.1% of original). ANT 17 steadily decreased over five cycles to 8×10^4 CFU/mL (0.004% of the original) until a final increase and leveling out at 2×10^7 CFU/mL (1.0% of original). WW3 decreased over three cycles with a slight increase to level out at approximately 3×10^7CFU/mL (11% of original).

To account for the possibility that growth was occurring on residual nutrients within the cryovial during the thaw portion of the cycle, vials from *E. coli*, ANT 17 and WW3 were enumerated at cycle 5 upon the beginning and at the completion of the 24-h thaw period. An increase in cell number was observed during the 24-h thaw period from 4.3×10^7 to 5.5×10^7 CFU/mL and 1.7×10^6 to 1.4×10^7 CFU/mL for *E. coli* and WW3 respectively. The data for ANT 17 was unfortunately unavailable due to experimental problems with the medium, however it is speculated that a similar pattern of regrowth might have occurred. The regrowth during the thaw period may affect the experimental outcome by artificially elevating the survival rate of the organisms tested. The presence of nutrients in the medium would provide material for growth. In retrospect, it would have been more insightful to utilize phosphate buffer as the medium in which the organisms were frozen. The presence of nutrients in the TSB may also be protective. This would maintain high osmolarity of the surrounding medium, allowing the organisms to expunge water to prevent intracellular freezing.

It appears that, under the test conditions used, the behaviour of ANT 17 is not unique to surviving cycles of freezing and thawing, as all three organisms decreased to a point until no further loss of cells was observed. Perhaps if the experiment were allowed to proceed for further cycles, the eventual loss of all organisms could be obtained. This may be more useful for determining the long-term survivability. It would be interesting to repeat the experiment utilizing soil microcosms, which could more closely parallel

conditions found *in situ*, to determine the survivability of ANT 17 compared to organisms from temperate sources.

3.3. Optimal temperatures for growth of ANT 17

3.3.1. Qualitative screening of temperatures permitting growth

ANT 17 was grown on PCA and incubated at different temperatures to qualitatively assess the growth temperature range of ANT 17. Table 3.4 summarizes the results obtained for ANT 17 as well as for *S. paucimobilis* WW3 (Section 3.3.3). Growth was observed at 1°, 4°, 8°, 15°, 22°, 28° and 30°C but no growth was observed at 35° or 37°C. These results served as a basis for further investigation of a more quantitative nature.

3.3.2. Growth rate analysis at different temperatures

ANT 17 was grown in liquid cultures incubated with shaking at 1°, 4°, 10°, 15°, 22°, 28°, 30° and 35°C (experiments from 4° to 35°C kindly conducted by Sara Ebert). Both QTSB and Bushnell-Haas with JP8 fuel supplied as the sole carbon and energy source were utilized. QTSB was selected as a model nutrient medium that is still dilute enough to not be too rich, as ANT 17 was isolated from an oligotrophic environment. JP8 fuel was chosen as ANT 17 was isolated in soil contaminated with this fuel. Cultures were monitored for growth using OD_{600} readings for QTSB medium or BCA assays for JP8 medium. Growth rates were determined (from the slope of the linear portion of the growth data plotted logarithmically) and normalized to generations/h in order to compare ANT 17 growth on the two different media directly, as different measurements were used to quantify growth. These values are plotted for each culture condition at each temperature (Figure 3.3).

The temperature optimum for ANT 17 appears to be approximately 28°C. Growth occurred on both media up to 30°C. Growth of ANT 17 did occur at 35°C in QTSB, unlike the qualitative screening method in Section 3.3.1. This anomaly is possibly due to the difference in growth on solid vs. liquid medium. Perhaps the cells had a higher temperature tolerance in liquid medium with shaking due to increased aeration. To summarize, ANT 17 can grow at cold temperatures in both dilute nutrient medium (QTSB) as well as on a refined petroleum product in mineral medium. Growth rates

Table 3.4. Qualitative assessment of ANT 17 and WW3 growth on PCA compared at different temperatures.

Temperature (°C)	ANT 17	WW3
1	+[a]	nt[c]
4	+	-
8	+	-
15	+	+
22	+	+
28	+	+
30	+	+
35	-[b]	+
37	-	+

[a] + indicates growth
[b] – indicates no growth
[c] nt indicates condition not tested

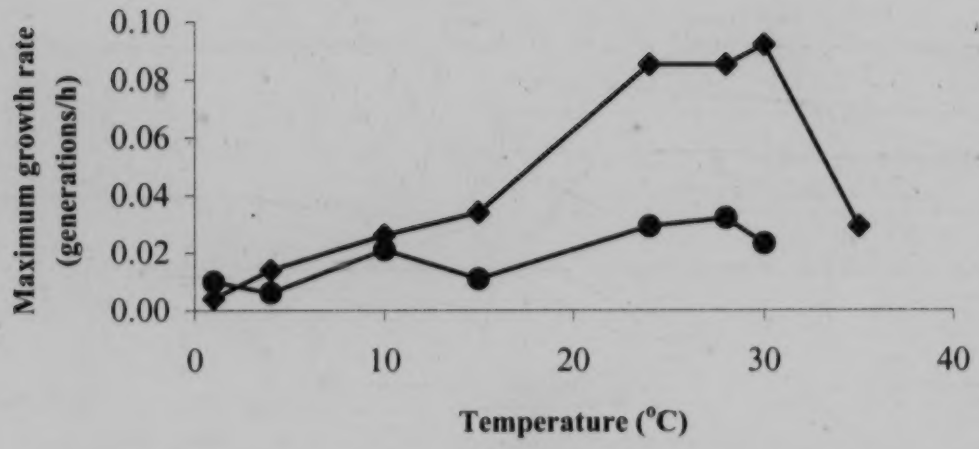

Figure 3.3. Growth rate of ANT 17 determined at temperatures from 1° to 35°C. ANT 17 was grown on QTSB (◆), measuring OD_{600}, or Bushnell-Haas with JP8 as the sole C source (●), measuring protein as µg BSA equivalents/mL.

were lower when ANT 17 was grown on the refined petroleum product compared to QTSB, until 10°C, at which growth rates were nearly identical.

Although ANT 17 is not a true psychrophile (growth temperature optimum at ≤ 15°C and maximum of 20°C), it is psychrotolerant (growth at low temperatures with temperature optimum above 20°C; Brock *et al.*, 1994). A mesophile is defined as an organism that grows best between 20° and 45°C, but typically not below 15°C. This temperature profile is not surprising, as the soil surface temperature in Antarctica can reach 20°C during the summer months. This is most likely the period when ANT 17 would be actively growing and degrading *in situ*. Whyte *et al.* (1999) have noted that in a fuel contaminated site from the high Arctic, there were more psychrotrophs (psychrotolerant organisms) than psychrophiles, and both of these types of organisms were more prominent than mesophiles. Vincent (1988) has noted that true psychrophiles are relatively uncommon within Antarctic terrestrial and freshwater environments and many of the psychrophiles isolated from these regions have enzyme optima at much higher temperatures. Bowman *et al.* (1997) isolated a variety of psychrophiles and psychrotrophs from Antarctic sea ice and sea water, however, all of the *Sphingomonas* spp. isolated were psychrotolerant.

The shallow growth rate curve with a refined petroleum product may indicate that biodegradation of these compounds may be nearly as efficient at colder temperatures as it is at its apparent optimum of 28° to 30°C. The apparent optimum may only be relevant for ANT 17 growth on nutrient medium under laboratory conditions. The ability of ANT 17 to grow on hydrocarbons at low temperatures is promising with respect to *in situ* applications. Under low temperatures the volatility of fuel contaminants is reduced, increasing their persistence. The ability of ANT 17 to grow and potentially degrade under this condition of decreased volatility and increased toxicity is promising. It would be interesting to analyze the cellular adaptations of ANT 17 under cold conditions while growing on hydrocarbons, such as differences in proteins being synthesized (such as cold-shock or cold-tolerant enzymes) as well as changes in the fatty acid profile, membrane fluidity and sphingolipids, as discussed in Section 3.1.1.8. In addition it would be interesting to determine if ANT 17 can grow and degrade at temperatures below zero. Given the shallow growth rate profile at the colder temperatures, and the fact that

Antarctic soils are subject to rapid changes in temperatures from just below to just above freezing, often several times daily, it would not be surprising to see growth at temperatures just below the freeing point, especially in soil where microsites with liquid water could be present at bulk soil temperatures below zero.

3.3.3. Comparison with *Sphingomonas* sp. WW3

S. paucimobilis WW3 was grown on PCA at 4°, 8°, 15°, 22°, 28°, 30°, 35° and 37°C. Table 3.4 (page 72) shows the results of this qualitative comparison. The differences in the growth temperatures of each organism can be most easily explained by adaptation to the temperatures of the sites each organism was isolated from. Strain WW3 was isolated from a temperate soil, so it seems reasonable that growth is observed from 15° to 37°C, characterizing WW3 as a mesophile. ANT 17, isolated from a cold climate soil, grows from 1° to 30°C justifying its classification as a psychrotolerant organism.

An example of organisms from the same genus exhibiting different patterns of growth and adaptation was found in a study by Chauhan and Shivaji (1994). Growth and carotenoid pigmentation of a psychrotrophic isolate, *Sphingobacterium antarcticus*, was compared to a mesophilic *Sphingobacterium* isolate. The psychrotrophic isolate exhibited an increase in pigmentation with increasing temperature, while the mesophilic isolate displayed the opposite result. It was thought that this was an adaptation to stiffen the membrane of the psychrotroph at higher temperatures, but this was not so. It would be enlightening to compare ANT 17 to other mesophilic *Sphingomonas* spp. including WW3 to determine the difference in cellular adaptations of these organisms to different temperatures, as outlined in Section 3.3.2.

3.4. Optimal pH for growth of ANT 17

These experiments were conducted to determine the pH range suitable for growth of ANT 17. The soil from which ANT 17 was isolated is quite alkaline (pH 8.1 to 9.6; Appendix A.3.3) and it is important to know whether or not this is optimal for ANT 17 growth.

3.4.1. Qualitative analysis of pH optimum

Tubes of NBG broth were adjusted to a final pH of approximately 3, 5, 6, 7, 8, 9, or 11 and used to qualitatively screen the range over which ANT 17 could grow. The true pH values were determined after autoclaving. ANT 17 was inoculated from a culture grown in neutral pH NBG. OD_{600} readings were used to compare turbidity after 2 days incubation at 22°C. Due to the differences in final volume after pH adjustment and each tube receiving the same inoculum, OD readings were expressed as to OD_{600} per mL. Growth was observed at pH 6.4, 6.8, 6.9, and 7.8 but not at 3.4, 3.9, 5.1, 8.6, 9.0, or 10.2. Figure 3.4 illustrates these results. This screen served to limit the range of pH values for quantitative testing.

3.4.2. Quantitative assessment of growth at various pH values

To quantify ANT 17 growth over a range of pH values, two systems were used to buffer QTSB (Section 2.1.4). Phosphate buffers are considered suitable from pH 5.8 to 8.0 and Tris-HCl from 8.0 to 9.0. However, the Tris-HCl buffer appeared to be toxic to ANT 17 as growth did not occur in cultures utilizing this buffering system (comparing pH 8 results of each buffering system, growth occurred with phosphate buffer but not with Tris-HCl). In other studies (conducted by S. Ebert) triethanolamine was used to achieve buffering in the alkaline pH ranges, but ANT 17 utilized this buffer as a C source which therefore was not suitable for comparison. Until a more suitable buffering system can be determined, ANT 17 growth at alkaline pH values cannot be assessed. The remaining quantitative studies employed the phosphate buffering system only, between pH 5.8 and 8.

An initial experiment utilized QTSB buffered from pH 5.9 to 7.8 with phosphate. Cultures were inoculated from an unbuffered TSB culture, incubated at 22°C in the dark with shaking and OD_{600} values were obtained. A pH 7.0 sterile control was included. From this experiment it was noted that ANT 17 grew at similar growth rates and with similar lag time between pH 5.9 and 6.6 (Figure 3.5a). At pH 6.8 to 7.8 the lag times increased steadily and dramatically, while the growth rates changed slightly (Figure 3.5b). These two figures are presented separately for clarity. The final pH of all

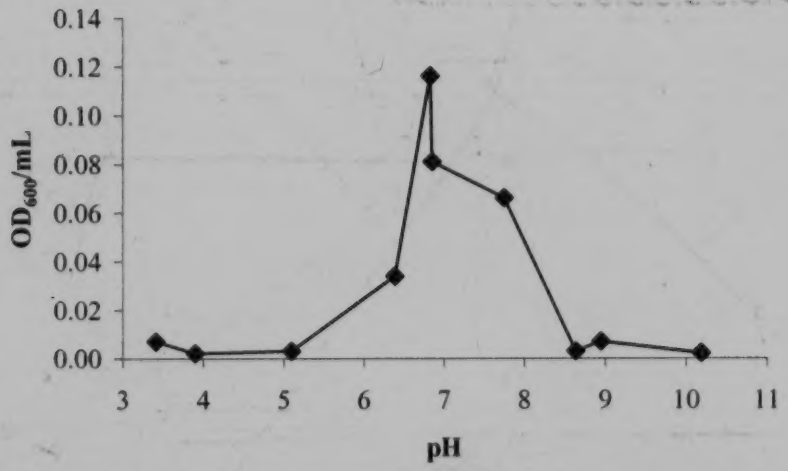

Figure 3.4. Qualitative estimate of the pH optimum for growth of ANT 17 in NBG adjusted pH values from 3.4 to 10.2 prior to inoculation. OD_{600} values were read after 2 days of incubation at 22°C.

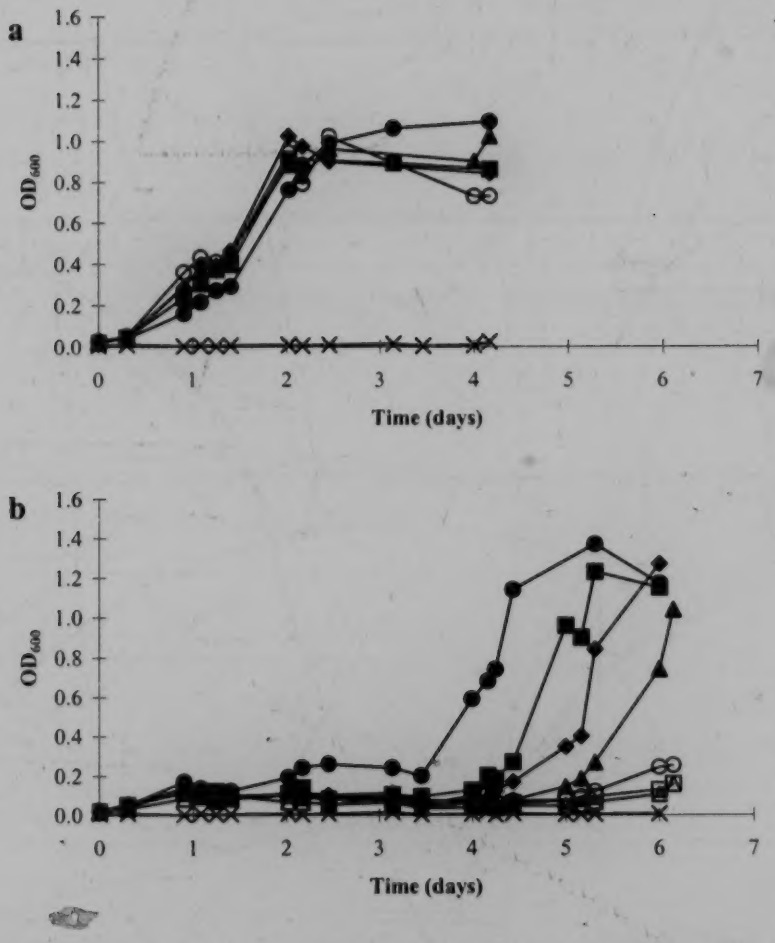

Figure 3.5. Growth of ANT 17 at 22°C in QTSB buffered between pH 5.9 to pH 7.8, as measured by OD_{600}. **a.** pH 5.9 (●), pH 6.0 (■), pH 6.2 (◆), pH 6.4 (▲), pH 6.6 (○), and pH 7.0 sterile control (×). **b.** pH 6.8 (●), pH 7.0 (■), pH 7.2 (◆), pH 7.4 (▲), pH 7.6 (○), pH 7.7 (□), pH 7.8 (△), and pH 7.0 sterile control (×).

cultures was assessed and each had remained within 0.3 pH units over the duration of the experiment.

The experiment was repeated utilizing the BCA assay to quantify ANT 17 growth over the same range of pH values. The BCA assay was employed in this case to obtain more accurate readings by avoiding a cell-clumping phenomenon observed previously. ANT 17 was again grown in QTSB, incubated at 22°C, with the inoculum prepared in unbuffered TSB. Figure 3.6 illustrates the results which agreed with the previous study (Figures 3.5a and 3.5b). Therefore the cell-clumping phenomenon did not seemingly affect the previous results. In addition, the final pH values all cultures remained within 0.3 pH units, except for the pH 7.2 culture which increased 0.5 pH units. Acidification of the culture medium was not observed to occur, therefore ANT 17 did not lower the alkaline medium to a more optimal pH. Growth rates and lag time were plotted to determine the pH optimum for ANT 17 (Figure 3.7a and 3.7b). From these figures, pH 6.4 appears to be the optimum for ANT 17 growth at 22°C on QTSB.

The apparent pH optimum of 6.4 was not expected, considering the alkaline pH of the soil where ANT 17 was isolated (pH 9.0). Assuming that ANT 17 can grow readily *in situ* on the hydrocarbon contaminants present under the alkaline conditions found there, it opens up the possibility for unique adaptations of ANT 17 to this environment. This study was conducted under laboratory conditions (liquid, dilute nutrient medium, ambient, and controlled temperatures, etc.). Perhaps the pH optimum for ANT 17 is not pH 6.4 under *in situ* conditions (cold soil, nutrient limitation). Further studies, with the availability of an adequate buffering system, might be able to address this phenomenon. An alternative is that perhaps ANT 17 alters its microsites *in situ* to a more suitable pH nearer its apparent optimum of 6.4. Both possibilities offer the promise of interesting research.

3.4.3. Growth at various pH values under cold conditions

To determine if the long lag times observed at alkaline pH values at 22°C would also occur at colder temperatures, growth of ANT 17 was determined as OD_{600} at 8° and 22°C in QTSB buffered at pH 6.4 and 7.6. Figure 3.8a illustrates these results. Although pH 6.4 permitted superior growth compared to pH 7.6 at 22°C, the results at 8°C

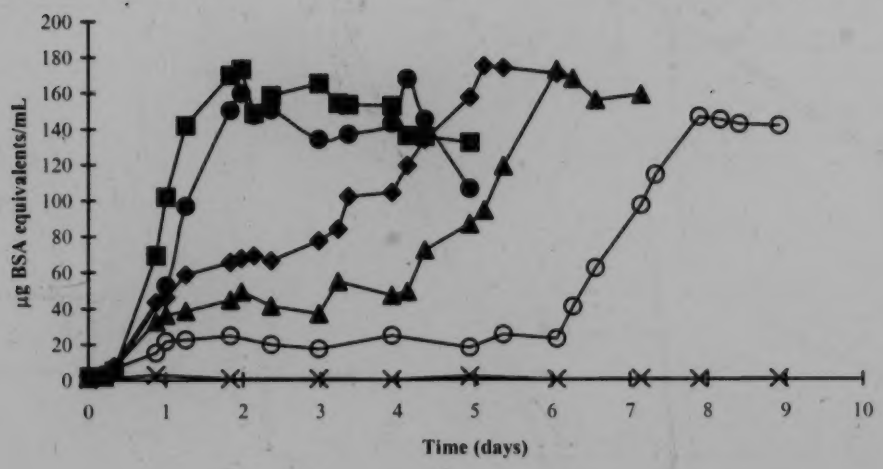

Figure 3.6. Growth of ANT 17 at 22°C in QTSB buffered from pH 6.0 to pH 7.8, as measured by protein concentration in µg BSA equivalents per mL. pH 6.0 (●), pH 6.4 (■), pH 6.8 (◆), pH 7.2 (▲), pH 7.8 (O), and pH 7.0 sterile control (×).

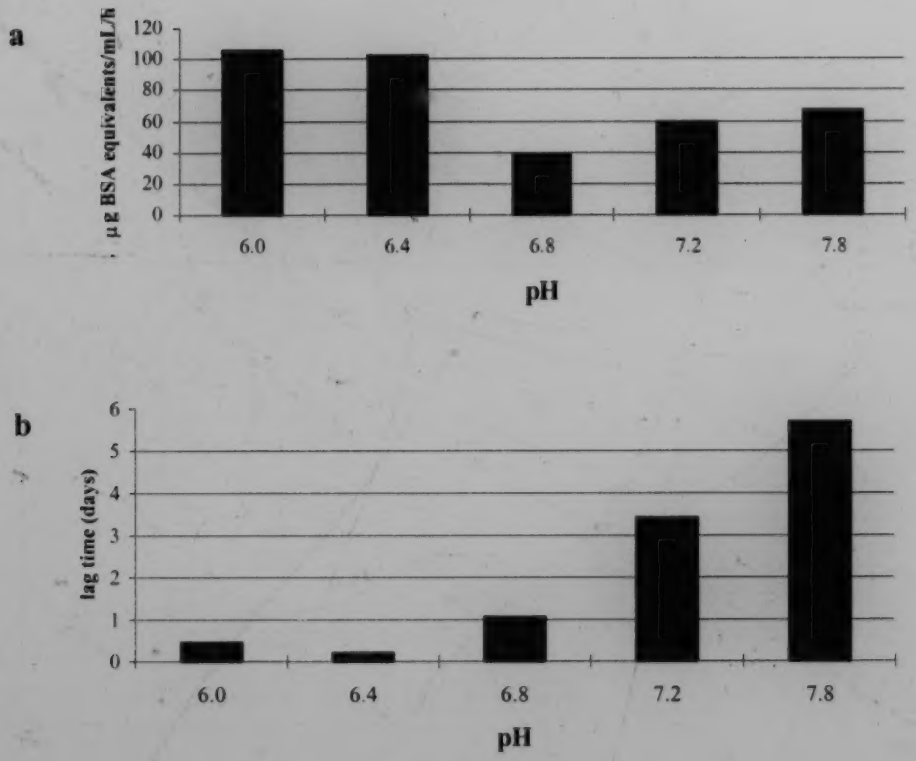

Figure 3.7. Growth rates and lag times compared from ANT 17 growth at 22°C in QTSB buffered from pH 6.0 to 7.8, as measured by protein concentration (data derived from Figure 3.6). **a.** Growth rate as calculated from the linear portion of growth after the lag phase. **b.** Lag time in days before logarithmic growth at each pH condition.

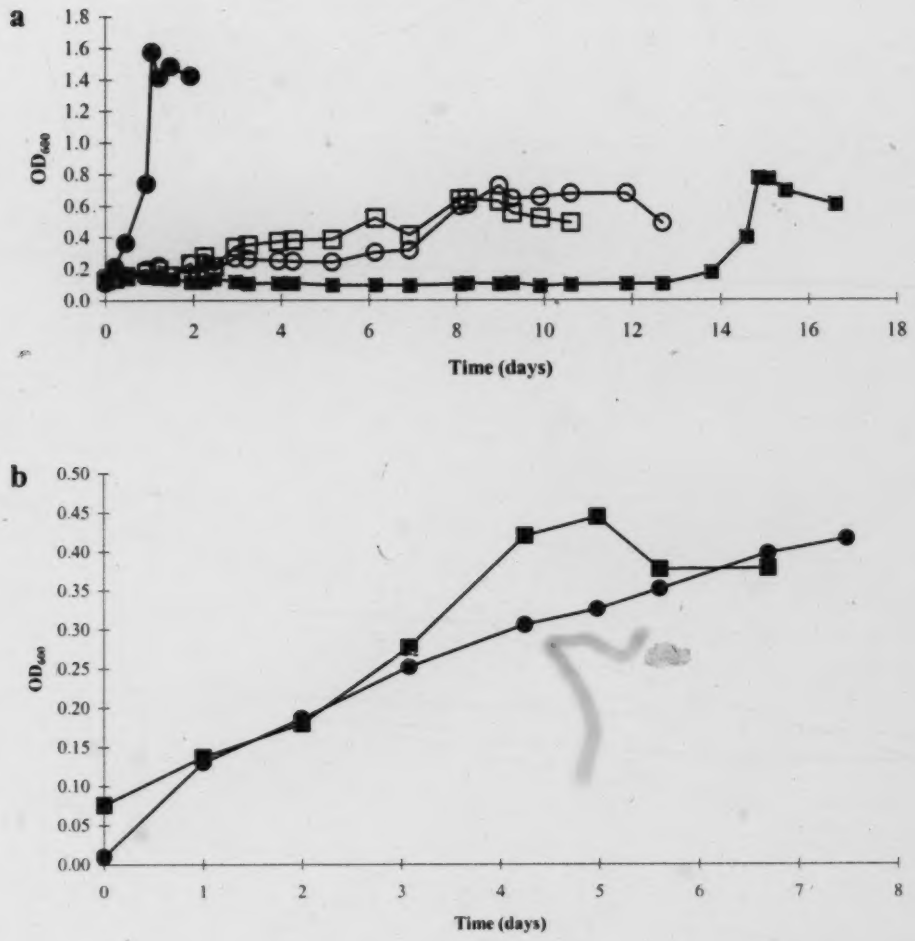

Figure 3.8. ANT 17 growth at various temperatures in QTSB buffered at pH 6.4 to pH 7.6. **a.** Growth at pH 6.4 (●) and pH 7.6 (■) at 22°C and pH 6.4 (○) and pH 7.6 (□) at 8°C. **b.** Growth at pH 6.7 (●) and pH 7.3 (■) at 4°C.

demonstrated that growth of ANT 17 was less sensitive to pH at the colder temperature. The lag times and growth rates at both pH values were nearly identical at 8°C, whereas at 22°C, the lag times were dramatically different. ANT 17 was also grown at 4°C at pH 6.7 and 7.3 (Figure 3.8b). Again, similar growth rates were observed at both pH values. The pH was assessed at the end of both experiments and did not change more than 0.4 pH units.

The implications of this are that perhaps ANT 17 is more pH tolerant in a cold environment such as in the soil from which it was isolated in Antarctica. Therefore, the highly alkaline pH of the soil might not be an important factor at these temperatures for ANT 17 growth and hydrocarbon biodegradation.

3.4.4. Comparison with *Sphingomonas* sp. WW3

In parallel experiments, ANT 17 and WW3 were grown in QTSB buffered at pH 6.4 and 7.6 at 22°C. Figure 3.9 illustrates the results. Both organisms exhibited similar growth at pH 6.4, while at pH 7.6, ANT 17 grew after a 12-day lag period and WW3 did not grow over the 21 days of the experiment. The pH of the medium was assessed at the end of the experiment and did not change more than 0.4 pH units. Although these results seem strange and unexpected, three other attempts were made to grow WW3 at pH 7.4 and 7.6. This organism did not grow under the conditions tested. Other buffering systems were not assessed. Weissenfels *et al.* (1990) assessed the pH optimum of WW3 when grown on phenanthrene at 30°C. The optimum pH was 7.2, however Tris was used as the buffering system for pH values from 7 to 9, while phosphate buffering was used for pH values from 5 to 7. Perhaps growth at the more alkaline pH is an adaptation of ANT 17, possibly in response to the alkaline nature of its isolation site. It would be useful to compare the pH optimum for ANT 17 to other environmental isolates of *Sphingomonas* spp. to determine the true nature of the observed growth patterns at various pH values.

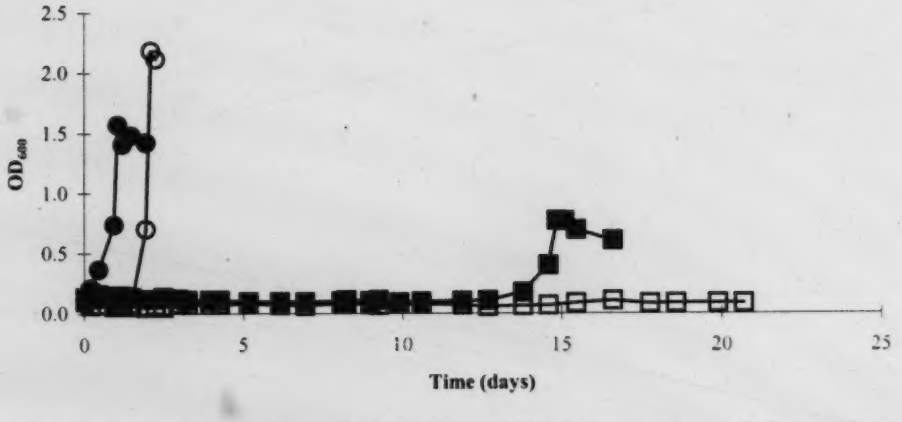

Figure 3.9. Comparison of ANT 17 and WW3 growth at 22°C in QTSB buffered at pH 6.4 and pH 7.6. ANT 17 pH 6.4 (●), ANT 17 pH 7.6 (■), WW3 pH 6.4 (○), and WW3 pH 7.6 (□).

3.5. Crude oil and refined product degradation

This portion of the research was conducted to evaluate the ability of ANT 17 to degrade crude oil and refined products under growth-limiting conditions such as low temperature, reduced aeration and differences in nutrient amendments.

3.5.1. Degradation of Prudhoe Bay crude oil at various temperatures

An investigation of Prudhoe Bay crude oil (PBCO) biodegradation at temperatures of 8°, 15°, 22°, and 28°C was conducted. Parallel cultures containing or lacking nutrient amendments (+ N,P or – N,P; composed of potassium nitrate, ammonium chloride and potassium phosphate; see Appendix B.3) were incubated at each temperature condition. Four hundred microlitres of oil were added to each flask. ANT 17 inoculum was prepared from a washed TSB seed culture grown at 22°C. Uninoculated controls were included at each temperature to assess abiotic losses (i.e. volatilization, dissolution, adsorption). Cultures were analyzed at time zero, 2 weeks (22° and 28°C conditions) or 4 weeks (8° and 15°C conditions) by extracting, fractionating and analyzing the oil by GC, as described (Section 2.8). A loopful of inoculum and each culture flask was streaked onto PCA plates, prior to acidification, to assess purity of the inoculated cultures and sterility of the uninoculated controls.

Figures 3.10a and b present gas chromatographic profiles of PBCO saturated and aromatic fractions at time zero. Some of the individual peaks and peak groups are identified for reference purposes (Fedorak and Westlake, 1981). The addition of the extraction standards (Appendix E.2) results in the presence of a squalane (sq) peak in the saturate fraction and an *o*-terphenyl (ter) peak in the aromatic fraction. Abiotic losses in incubated cultures can be seen by comparing sterile control profiles to the time zero profiles.

Figure 3.11 illustrates representative results observed for the PBCO saturated fraction after 4 weeks incubation at 8°C: a sterile control (Figure 3.11a) and an inoculated culture containing N,P nutrient amendment (Figure 3.11b). Abiotic losses of volatile alkanes were observed as expected (comparing Figures 3.11a to 3.10a). By comparing the sterile control results (Figure 3.11a) to inoculated cultures it was noted that no degradation of the saturate fraction occurred under any of the temperature conditions.

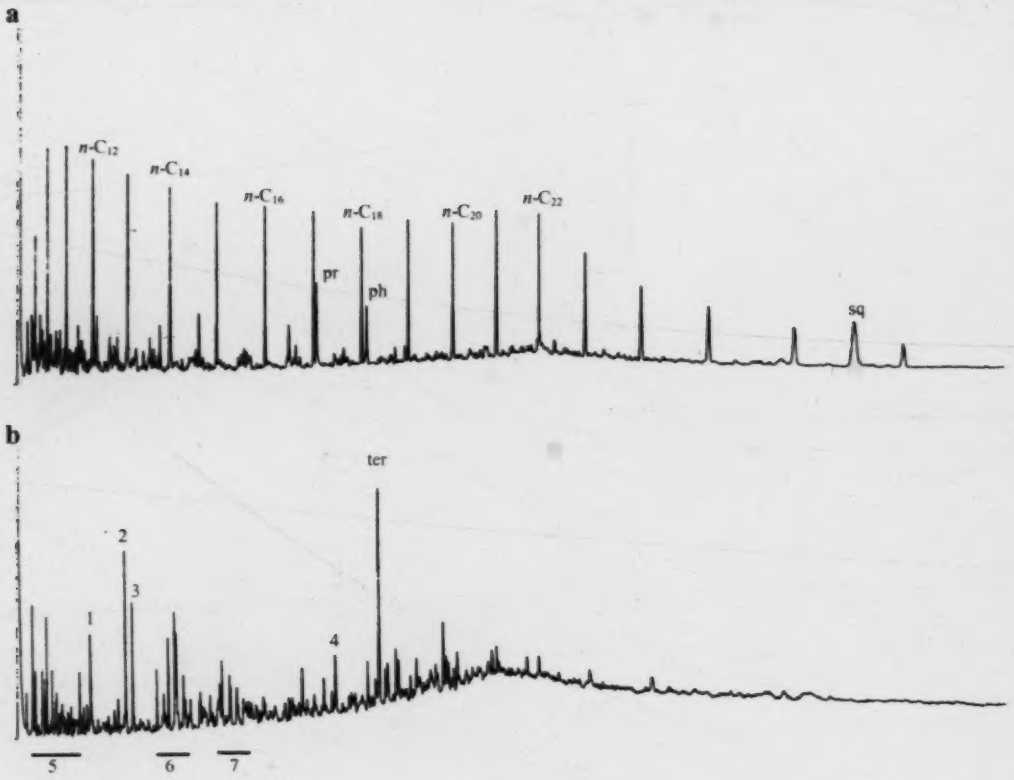

Figure 3.10. Gas chromatographic analysis of PBCO fractions at time zero. **a.** Saturated fraction with peak identification of even *n*-alkanes; pristane, pr; phytane, ph; and squalane (extraction standard), sq. **b.** Aromatic fraction with peak identification of naphthalene, 1; 2-methylnaphthalene, 2; 1-methylnaphthalene, 3; phenanthrene, 4; C_3-, C_4-, and C_5-benzenes, 5; C_2-naphthalenes, 6; C_3-naphthalenes, 7; and *o*-terphenyl (extraction standard), ter.

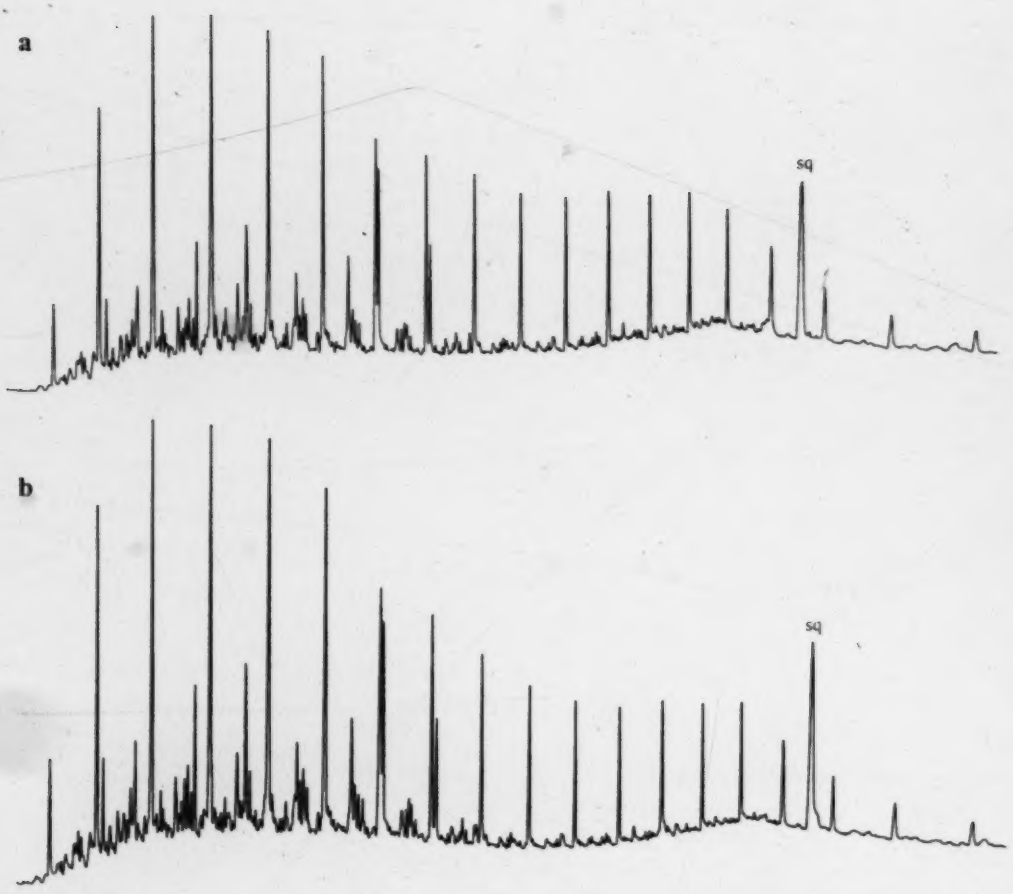

Figure 3.11. Gas chromatographic analysis of PBCO saturated fractions after 4 weeks incubation at 8°C. **a.** sterile control. **b.** ANT 17 culture containing N,P nutrient amendment. Squalane (extraction standard, sq). For other peak identifications refer to Figure 3.10a.

The saturated profiles for all inoculated cultures appeared identical to the sterile control profiles, thus ANT 17 did not appear to degrade the saturate fraction of PBCO. ANT 17 was originally isolated on 1-methylnaphthalene therefore degradation of the saturate fraction was not expected despite the fact that the JP8 contaminated soil contained significant quantities of *n*-alkanes (Appendix A.3.1).

Figure 3.12 compares representative results of biodegradation of the aromatic fraction. The profiles after 4 weeks incubation at 8°C are shown. Degradation is qualitatively observed as a reduction in peak height compared to the sterile control (all normalized to the surrogate extraction standards added to account for differences in extraction efficiency and detector attenuation). Peak height reduction or peak loss indicates transformation (oxidation, ring cleavage or mineralization) of a particular compound. A sterile control (Figure 3.12a) is presented for comparison with inoculated cultures lacking N,P nutrient amendment (Figure 3.12b) or containing N,P (Figure 3.12c). Abiotic loss of the volatile aromatic compounds (benzenes and some of the naphthalenes) was expected and evident by comparing Figure 3.12a to 3.10b. Figure 3.13 illustrates parallel results observed at 22°C after 2 weeks (with and without N,P; Figures 3.13b and c, respectively).

At all temperatures, degradation of the aromatic fraction of PBCO was observed. The warmer temperatures resulted in more complete degradation within a shorter time period, however the degradation occurring at the cold temperatures was still extensive. Perhaps longer incubation times at the colder temperatures would result in degradation as complete as that observed at the warmer temperatures. Degradation was slightly better in cultures containing the N,P nutrient amendment, however degradation was extensive in both cases. Due to the limitations of this analytical method, there is the possibility that degradation was more complete (mineralization) in the presence of nutrients, whereas degradation may have been incomplete (cometabolic oxidation and release of polar products) in the absence of nutrients. The extensive profile modification of the aromatic fraction of PBCO was not expected. ANT 17 was isolated on JP8 contaminated soil which contains mostly mono- and di-aromatics (Appendix A.3.1) which is narrower in content than crude oil. This would suggest that ANT 17 possesses a broad substrate range capable of transforming a wide variety of aromatic hydrocarbon compounds.

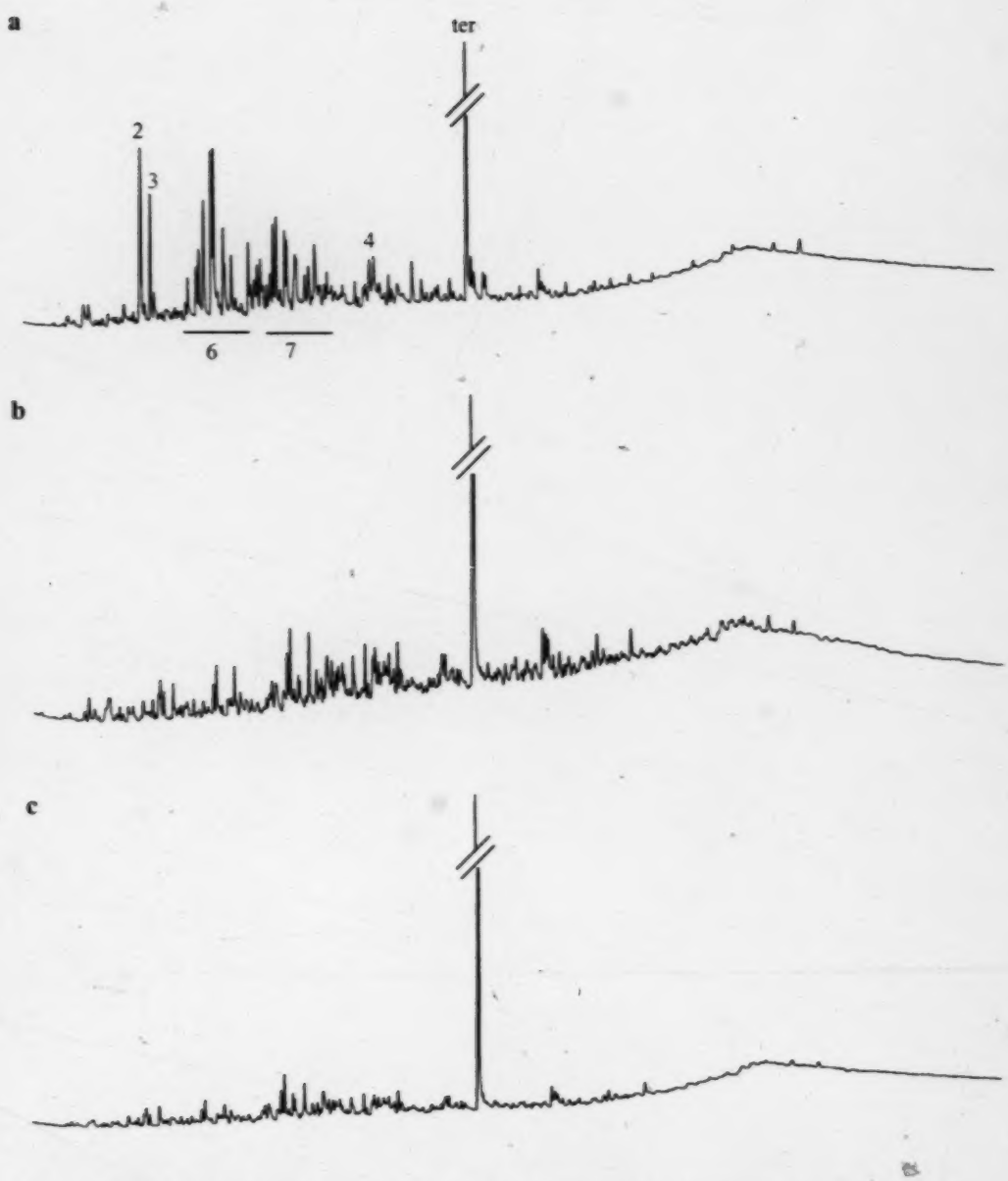

Figure 3.12. Gas chromatographic analysis of PBCO aromatic fractions after 4 weeks incubation at 8°C. **a.** sterile control. **b.** ANT 17 culture lacking N,P nutrient amendment. **c.** ANT 17 culture containing N,P nutrient amendment. *o*-Terphenyl (extraction standard, ter). For peak identifications refer to Figure 3.10b.

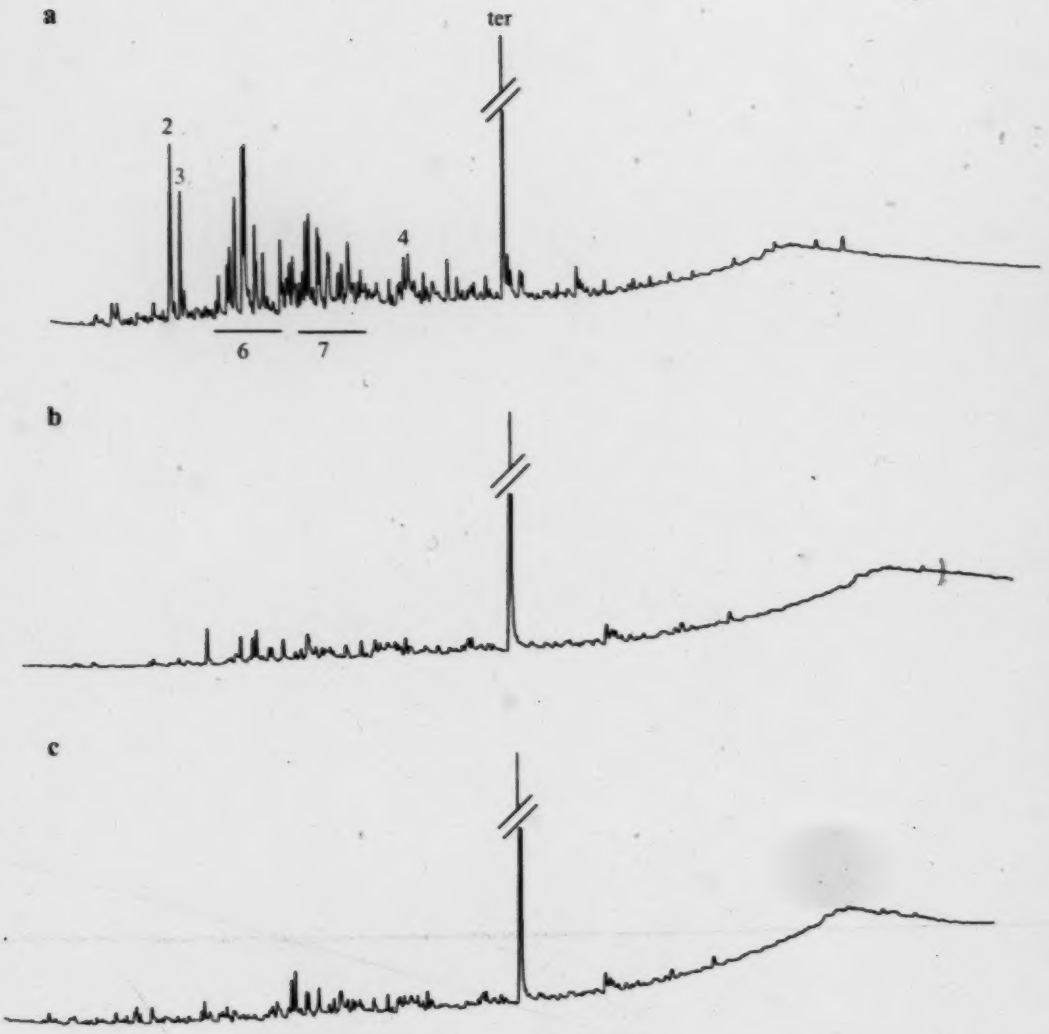

Figure 3.13. Gas chromatographic analysis of PBCO aromatic fractions after 2 weeks incubation at 22°C. **a.** sterile control **b.** ANT 17 culture lacking N,P nutrient amendment. **c.** ANT 17 culture containing N,P nutrient amendment. *o*-Terphenyl (extraction standard, ter). For peak identifications refer to Figure 3.10b.

Sphingomonas spp. with broad substrate ranges for many aromatic compounds are known, as discussed in Section 1.7.2.

3.5.2. Degradation of various oils and a refined product at cold temperatures

Once degradation of PBCO was established as occurring over a range of temperatures, degradation was assessed of various oils and a refined product. Different oils were tested due to their different compositions. In order to assess the broad substrate range of ANT 17, these different oils and products were tested: Alberta Sweet Mixed Blend crude oil (ASMB) and Norman Wells crude oil (NW) were selected as they are of interest to industry of Northern Alberta and the sponsor for this research; Diesel #2 (a refined oil product) is a widely used product with the potential for many spills; JP8 was not used for this analysis, as its volatility would have caused analytical problems.

ANT 17 inoculum was prepared from a 2-day old washed QTSB seed culture grown at 28°C. Cultures containing 200 µL of ASMB, NW, or Diesel #2 fuel were incubated in parallel with PBCO. Sterile controls and cultures were incubated with shaking at 10°C in the presence and absence of N,P nutrient amendments, and analyzed after 4 and 8 weeks.

A pink to orange colour and oil emulsification were observed at 4 weeks in the PBCO + N,P and – N,P cultures and the ASMB + N,P and – N,P cultures. The Diesel #2 – N,P and NW cultures were not coloured but were emulsified. By 8 weeks, the pink to orange colour had disappeared in all cultures except the Diesel #2 + N,P which became orange. The production of the orange colour by ANT 17, as observed in these cultures, suggests the transformation of one or more of the compounds within the oils and refined product. The eventual disappearance of the colour suggests that continued transformation of the coloured intermediates occurred.

GC-FID analysis of the fractionated extracts indicated that no saturate degradation occurred under any of the culture conditions (results not shown), confirming previous results (Section 3.5.1). The aromatic fraction profiles for the 4-week analysis (not shown) indicated that degradation had occurred to some degree in all cultures. Chromatograms of the aromatic fractions after 8 weeks incubation are shown in Figures 3.14 to 3.17. PBCO sterile control is compared to inoculated flasks – N,P and + N,P (Figure 3.14a, b and c respectively). Extensive degradation of the aromatic fraction was

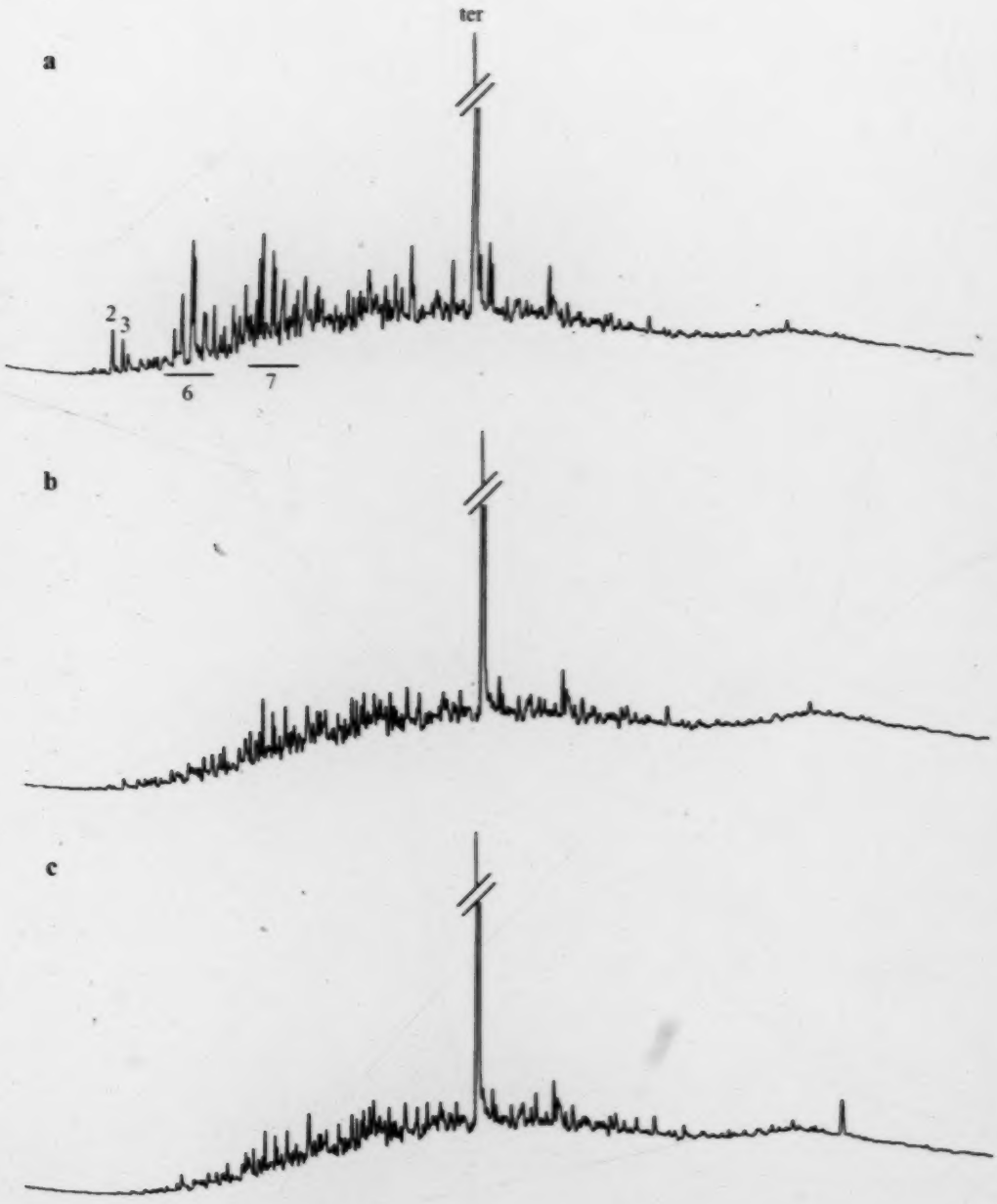

Figure 3.14. Gas chromatographic analysis of PBCO aromatic fractions after 8 weeks incubation at 10°C. **a.** sterile control. **b.** ANT 17 culture lacking N,P nutrient amendment. **c.** ANT 17 culture containing nutrient amendment. *o*-Terphenyl (extraction standard, ter). For peak identifications refer to Figure 3.10b.

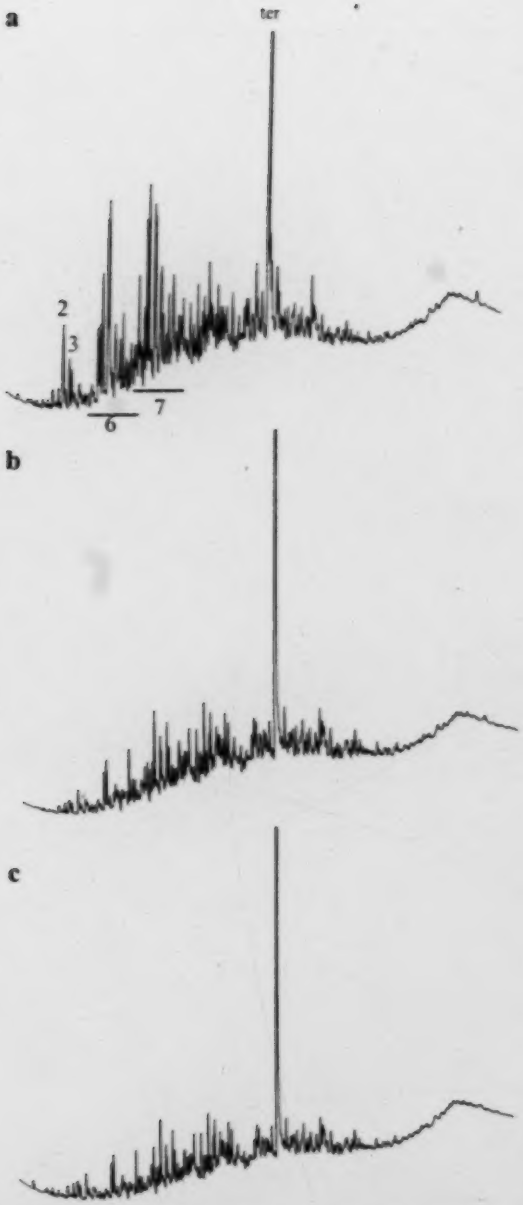

Figure 3.15. Gas chromatographic analysis of ASMB aromatic fractions after 8 weeks incubation at 10°C. **a.** sterile control. **b.** ANT 17 culture lacking N,P nutrient amendment. **c.** ANT 17 culture containing N,P nutrient amendment. *o*-Terphenyl (extraction standard, ter). For peak identifications refer to Figure 3.10b.

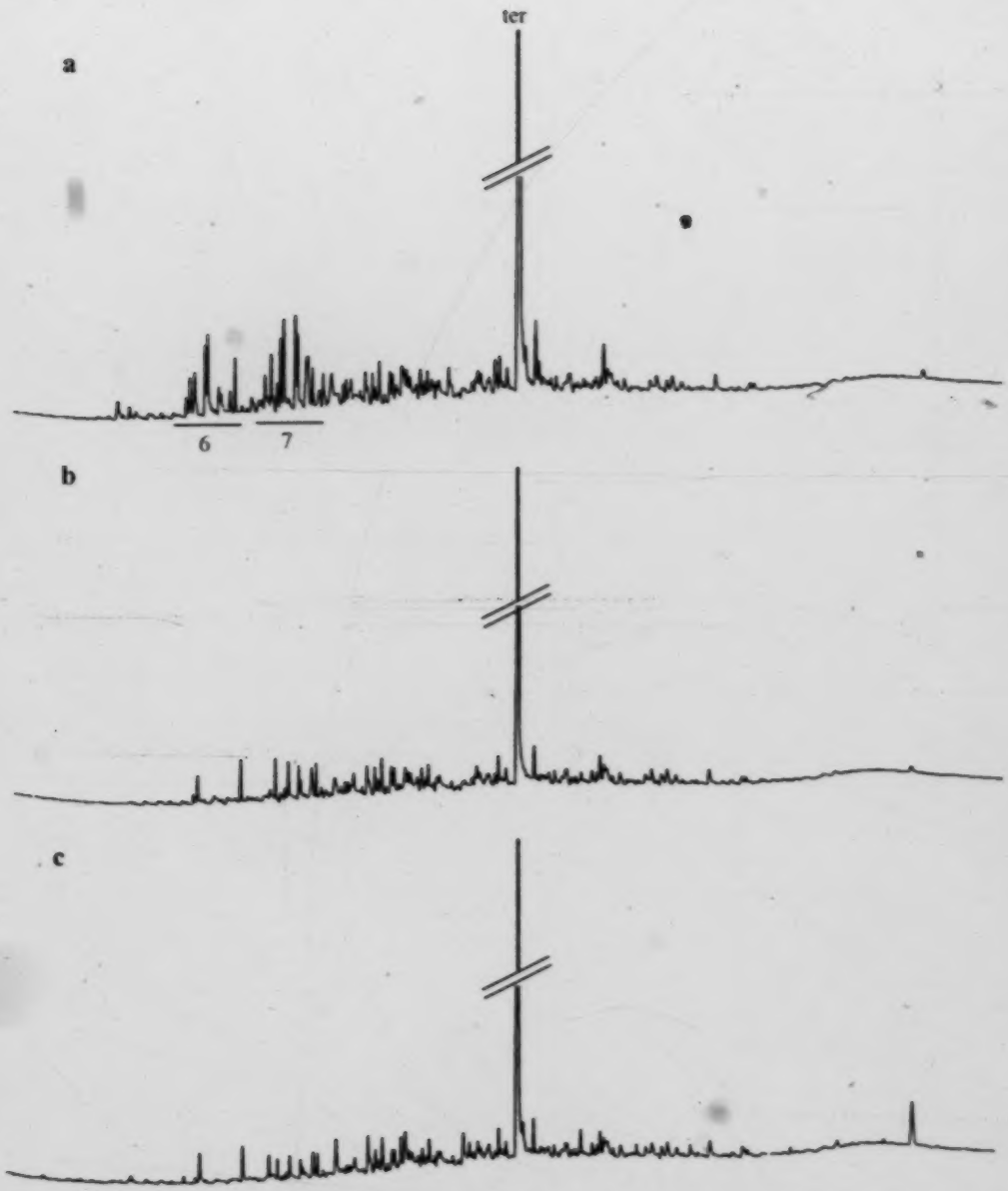

Figure 3.16. Gas chromatographic analysis of NW aromatic fractions after 8 weeks incubation at 10°C. **a.** sterile control. **b.** ANT 17 culture lacking N,P nutrient amendment. **c.** ANT 17 culture containing N,P nutrient amendment. *o*-Terphenyl (extraction standard, ter). For peak identifications refer to Figure 3.10b.

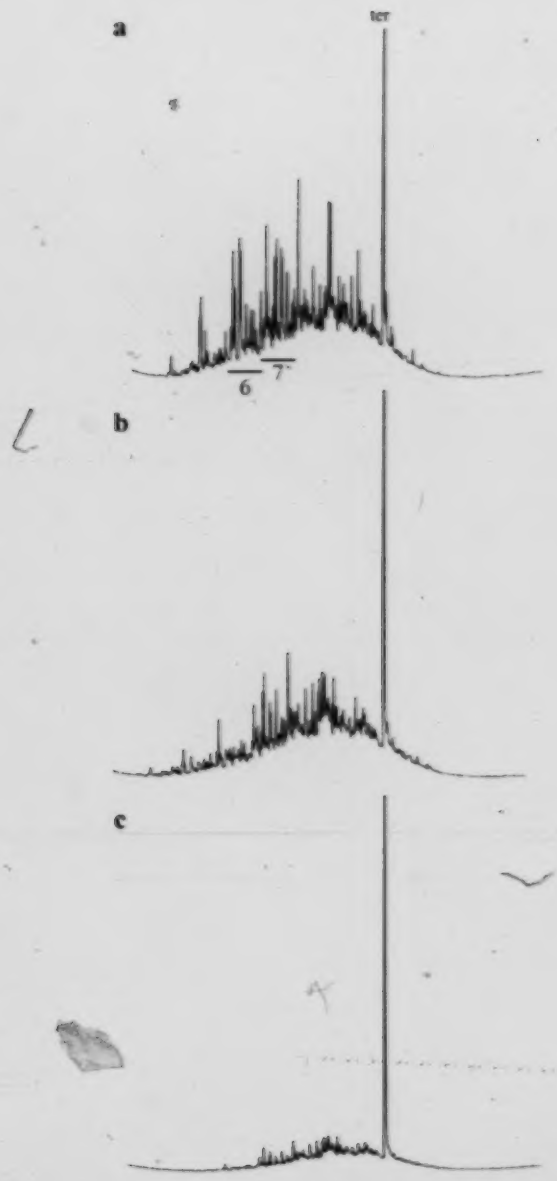

Figure 3.17. Gas chromatographic analysis of Diesel #2 aromatic fractions after 8 weeks incubation at 10°C. **a.** sterile control. **b.** ANT 17 culture lacking N,P nutrient amendment. **c.** ANT 17 culture containing N,P nutrient amendment. *o*-Terphenyl (extraction standard, ter). For peak identifications refer to Figure 3.10b.

observed in cultures containing and lacking N,P nutrient amendments. These findings were similar to those shown in Figure 3.12.

Figure 3.15 presents the results with ASMB that parallel those seen for PBCO, where degradation of the aromatic fraction has occurred with and without the N,P nutrient amendment. Biodegradation of the ASMB aromatics was not as extensive compared to PBCO aromatic biodegradation. This is most likely due to a compositional difference between the crude oils, but this phenomenon was not investigated further. The results for NW aromatic fraction degradation are also similar (Figure 3.16). Figure 3.16c shows slight saturate fraction contamination as indicated by the presence of the squalane peak at the end of the profile, however, degradation is evident again in the presence and absence of N,P nutrient amendments.

Degradation occurred with and without N,P amendment, with degradation more extensive in the presence of N,P (Figure 3.17). This is interesting in light of the colour observations where the –N,P culture turned orange prior to the +N,P culture, possibly indicating degradation was occurring more rapidly in the absence of N,P. Perhaps degradation was initially more rapid in the +N,P culture than in the –N,P culture, that at 4 weeks the +N,P culture was already at the stage seen at 8 weeks for the other cultures. The orange colour present at 8 weeks in the +N,P culture may be the result of further transformation of different compounds. Perhaps this is an example of diauxic growth where ANT 17 utilized or transformed a certain compound(s) first then attacked a more recalcitrant compound(s). This would explain the difference in the degradation observed in Figures 3.17b and 3.17c as well as the colour observation at 4 and 8 weeks.

ANT 17 appears to be quite versatile in its ability to degrade a variety of crude oils and a refined product each with different compositions. This is in addition to its apparent broad substrate diversity as discussed in Section 3.5.1. ANT 17 is also capable of degradation in both the presence and absence of nutrient amendments. This is unusual as many biodegradation studies under cold conditions required the supplement of nutrients for any significant biodegradation to occur (as described in Section 1.5). These observed results may reflect the isolation site of ANT 17 as it was lacking in nutrients, especially available N. ANT 17 apparently is very well adapted for biodegradation under cold and nutrient-poor conditions.

3.5.3. Effect of aeration and agitation on Prudhoe Bay crude oil degradation

PBCO degradation was compared after 2 and 4 weeks with and without gyrotory shaking at 28°C. Two hundred microlitres of PBCO was added to medium amended with N,P nutrients. A washed QTSB seed culture grown at 28°C was used as the inoculum. Figure 3.18 compares the results after 2 weeks incubation. The agitated (and thus more aerated) culture was only slightly more degraded than the non-agitated culture. After 4 weeks, the aromatic fraction degradation was the same (not shown). No saturate degradation was observed.

From these findings, aeration and agitation does not significantly increase PBCO degradation at the temperature optimum for ANT 17. This was unexpected as, in general, increasing the aeration of an aerobic culture and increasing available surface area of crude oil for dissolution or colonization through droplet formation in agitated cultures, increases the biodegradation. This is due to the initial oxidation reactions at the start of biodegradation requiring molecular oxygen. Generally these initial oxidations are considered the rate-limiting steps (Leahy and Colwell, 1990; Cerniglia, 1992). Perhaps there was enough oxygen in the headspace to allow an adequate amount to partition into the liquid medium. The oil in the static culture appeared as a slick on the top of the medium surface. If ANT 17 partitioned at the medium-oil interface in close proximity to the air-medium interface, oxygen may not have been limiting. In previous studies (Sections 3.5.1 and 3.5.2) as well in this case, the shaking cultures all appeared emulsified. If ANT 17 also has the ability to produce a biosurfactant, this would enhance the accessibility of the oil for biodegradation within the medium itself, where perhaps dissolved oxygen would not be limiting. Given that the degradation was in fact slightly slower at 2 weeks for the static culture condition, a slightly reduced oxygen concentration may have been rate limiting. Once the initial oxidation reactions were commenced, biodegradation of the aromatic fraction could proceed, resulting in similar profiles seen at 4 weeks for both static and shaking conditions. Further analyses of oil degradation at incubation times shorter than 2 weeks could be employed to determine if there is a significant difference with shaking than without, at the early stages of incubation.

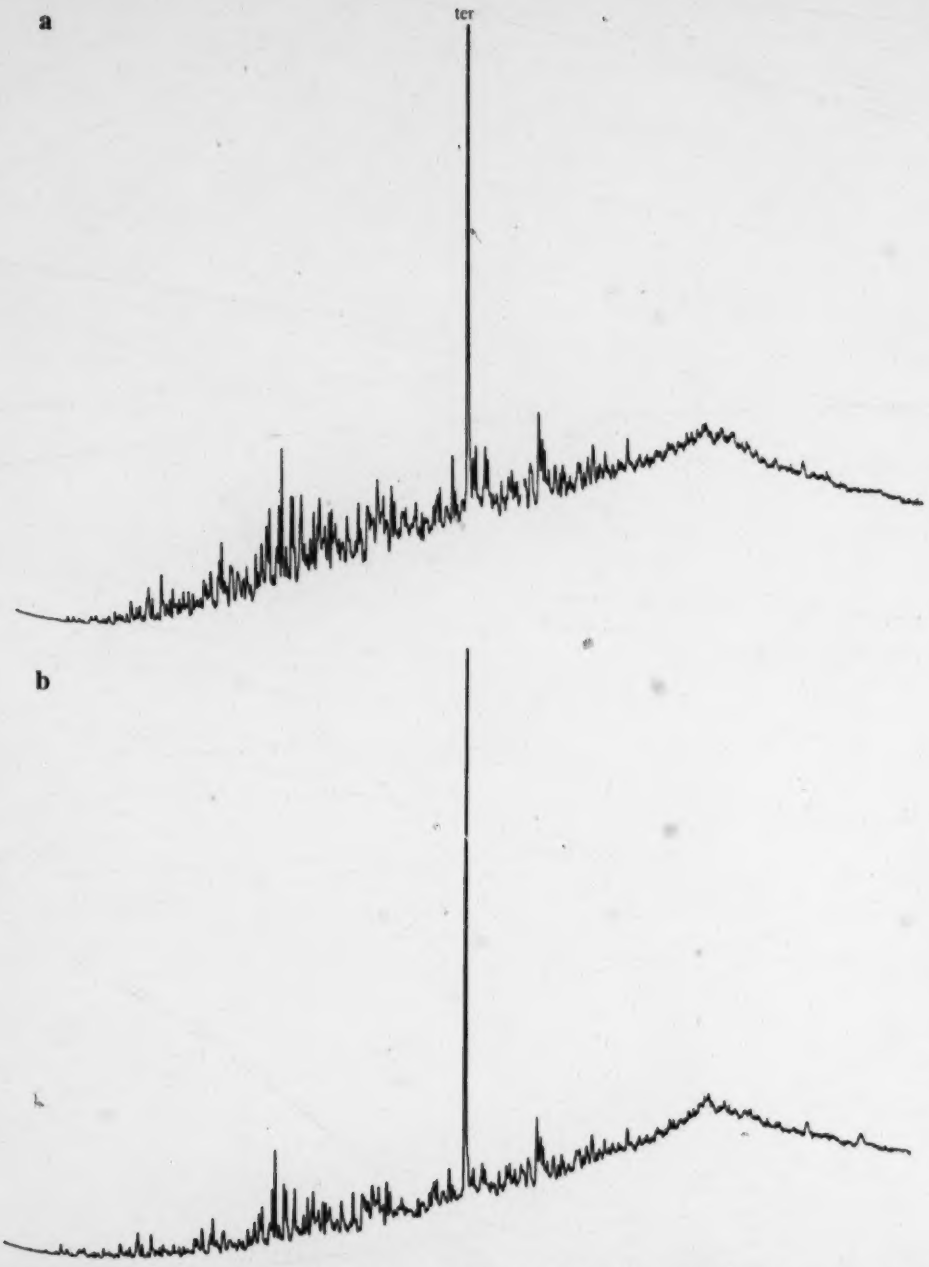

Figure 3.18. Gas chromatographic analysis of PBCO aromatic fractions after 2 weeks incubation at 28°C. ANT 17 cultures contained nutrient amendments and were incubated without gyrotory shaking (**a**) or with gyrotory shaking (**b**). *o*-Terphenyl (extraction standard, ter). For peak identifications refer to Figure 3.10b. For comparable sterile control profile of PBCO aromatic fraction refer to Figure 3.12a.

3.5.4. Norman Wells crude oil degradation at cold temperatures with various nutrient amendments

Foght *et al.* (1999) showed that the type of N source supplied as a nutrient amendment affected the extent of biodegradation of the aromatic fraction of crude oil by a bacterial consortium. To assess whether or not ANT 17 was similarly affected, an experiment was conducted with various types of N sources. ANT 17 inoculum was prepared from a washed QTSB culture and inoculated into parallel flasks of mineral medium containing 200 µL NW. Nutrient conditions were varied to include no nutrient amendment (-N,P), standard nutrient amendment (+N,P; 20 mM nitrate, 20 mM ammonium and 3 mM phosphate), phosphate only (P; 3mM phosphate), nitrate with phosphate (NO_3^-, P; 40 mM nitrate and 3 mM phosphate) and ammonium with phosphate (NH_4^+, P; 40 mM ammonium and 3 mM phosphate). The final concentrations of nitrate and ammonium were adjusted so that equimolar amounts of N were maintained (equal to standard N,P). The cultures were incubated at 10°C with shaking and analyzed after 2, 4, and 8 weeks.

The results after 8 weeks are presented in Figure 3.19. ANT 17 was not significantly affected by changing the nutrient amendment within the oil cultures, as the level of degradation of the NW aromatic fraction was constant. Saturate degradation was not observed in any of the test conditions. As ANT 17 was isolated from a N-poor site, it may be adapted to biodegradation in the absence of this nutrient. Therefore no observable net increase in the extent of biodegradation was observed with the inclusion of this nutrient. However, due to the limitations of this analytical method, it is unknown whether or not only transformation was occurring in the absence of N,P whereas growth was occurring in the presence of N,P.

In the research by Foght *et al.* (1999), the observed changes in aromatic degradation were noted with a consortium of aromatic- and saturate-degrading microorganisms. In the presence of ammonium, the saturate degraders acidified the medium and inhibited aromatic biodegradation. Nitrate did not cause these observed effects. ANT 17 did not acidify the medium under any of the tested conditions. The next course of research would be to include ANT 17 within a consortium of other saturate-degrading

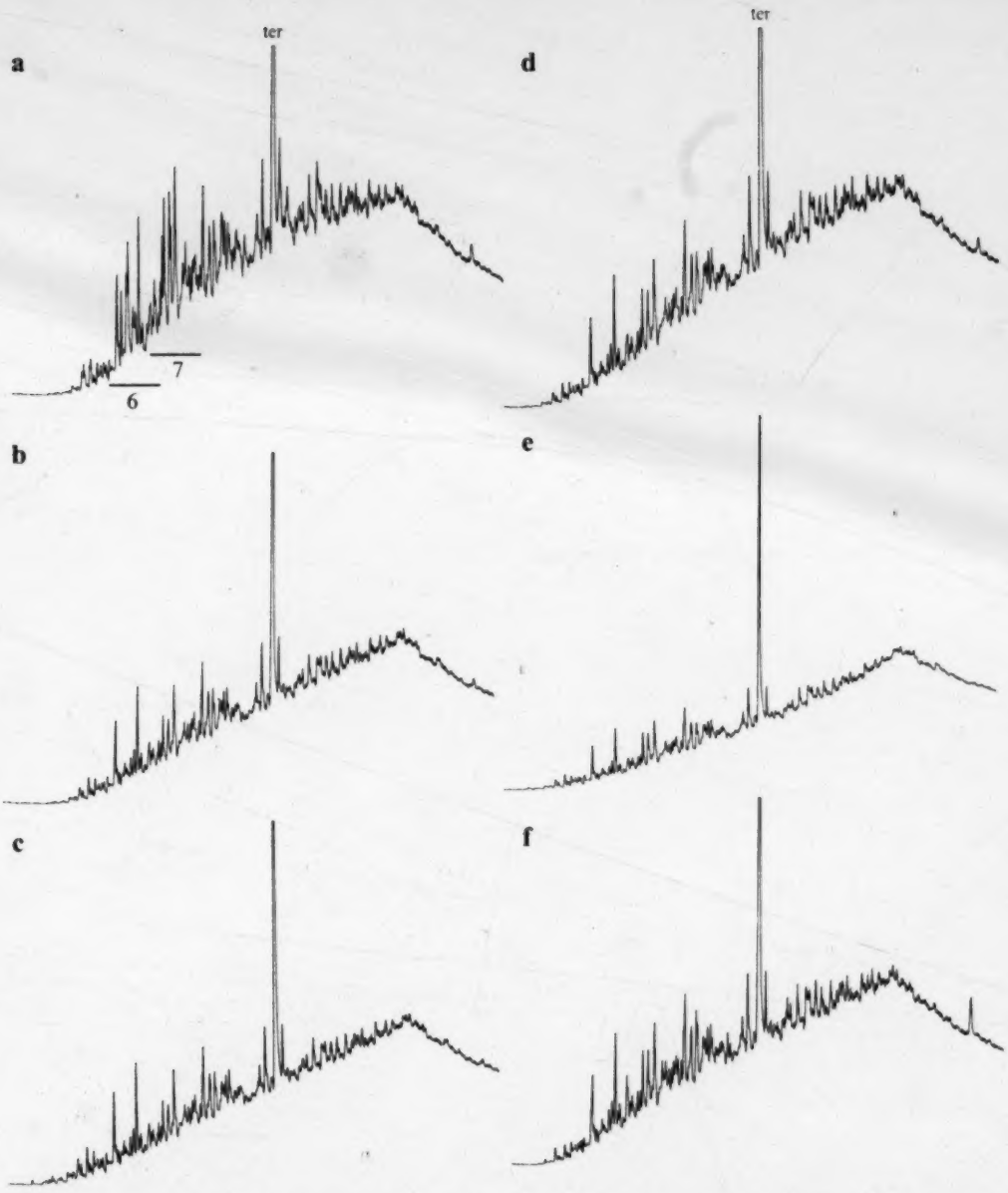

Figure 3.19. Gas chromatographic analysis of NW aromatic fractions after 8 weeks incubation at 10°C. Profiles shown; sterile control (**a**); and ANT 17 cultures –N,P (**b**); +N,P (**c**); P (**d**); NO_3^-, P (**e**); and NH_4^+, P (**f**). *o*-Terphenyl (extraction standard, ter). For peak identifications refer to Figure 3.10b.

microorganisms in order to determine if ANT 17 would be similarly affected by culture acidification by saturate degraders and changing the type of nutrient amendments.

3.6. Carbon and energy sources

This portion of the research was conducted to determine which compounds found in crude and refined oils can support the growth of ANT 17 as a sole C and energy source, or can be transformed.

3.6.1. Qualitative screening results

Qualitative screening of potential substrates was conducted utilizing various methods. Liquid cultures, vapour plates, spray plates, overlay plates, as well as plates with suspended test compounds were used as described in Section 2.1.3 and Appendix B.3. Table 3.5 summarizes the results. A variety of methods were employed in order to prevent the possibility that a compound might not support growth when supplied in one form compared to another (e.g. growth observed when substrate supplied in vapour phase but not in solid phase). This phenomenon may be due to the toxicity levels or the requirement for a physical interaction with the substrate. Compounds were selected on the basis of parent compounds, variations of the parent compounds as alkyl homologues, acids, alcohols, and heterocycles, as well as selected potential metabolites.

Growth was determined as colonies on a plate, with the test substrate sprayed, overlaid, suspended in the agar medium, or presented as vapours. Transformation was noted as the production of a coloured product either on the plate or within a liquid medium. Transformation of compounds to non-chromogenic metabolites would not have been observed by these methods, however solublization (a decrease in insoluble substrate) was observed without the presence of coloured products when some compounds were tested in liquid medium. Solublization was also visible around colonies on spray and overlay plates as a cleared zone within the insoluble (opaque) compound. These observations are noted in Table 3.5. In all cases a C-free control was included to ensure that ANT 17 growth was not artifact of the medium. In addition, uninoculated controls were included for the liquid culture tests to confirm that the

Table 3.5. Qualitative assessment of the ability of *Sphingomonas* sp. ANT 17 to transform or utilize 75 pure compounds, including hydrocarbons and heterocycles present in petroleum and their metabolites, determined using five different methods. Compounds are grouped according to chemical class, and ordered from simplest to most substituted. **"G"** signifies growth on the compound (utilization); **"T"** signifies transformation of the compound (oxidation without growth, generally determined by production of coloured metabolites); **"S"** indicates solubilization of the compound on spray plates or in liquid medium; **"±S"** indicates moderate solubilization; **"N"** indicates no observable metabolism, **"?"** indicates indeterminate metabolism, and blank spaces indicate conditions that were not tested.

Pure compounds	Assessment method used[a]					Interpretation of Results
	Vapour plate	Spray plate	Overlay Plate	Liquid medium	Dissolved substrate	
Aromatic hydrocarbons						
benzene	N					No Metabolism
m –xylene	G				N	Growth substrate in vapour phase
4-phenyltoluene	N			N	N	No Metabolism
biphenyl	N	T	N		N	Transformation
naphthalene	G		N	G	G	Growth substrate
1-methylnaphthalene	G			S		Growth substrate
2-methylnaphthalene	G					Growth substrate
1,2-dimethylnaphthalene		G		S		Growth substrate
1,3-dimethylnaphthalene		G		S		Growth substrate
1,4-dimethylnaphthalene		G		S		Growth substrate
1,5-dimethylnaphthalene				S		Solubilization
1,7-dimethylnaphthalene		G		S		Growth substrate
1,8-dimethylnaphthalene				S		Solubilization
2,3-dimethylnaphthalene		G		S		Growth substrate
2,6-dimethylnaphthalene		G				Growth substrate
2-ethylnaphthalene	G					Growth substrate
1-phenylnaphthalene				N		No Metabolism
2-phenylnaphthalene				S		Solubilization
anthracene		±S	±S	S		Solubilization
2-methylanthracene		S		T		Transformation
fluorene		G	N			Growth substrate
phenanthrene		G	G	G		Growth substrate
fluoranthene		±S	N	T	N	Transformation
triphenylene				S		Solubilization
pyrene		S	T	T		Transformation
chrysene		?		T		Transformation

[a] see Section 2.1.3 and Appendix B.3 for description of methods

Table 3.5. (continued)

Pure compounds	Vapour plate	Spray plate	Overlay Plate	Liquid medium	Dissolved substrate	Interpretation of Results
Aromatic alcohols, aldehydes, ketones and quinones						
catechol		?		?		Undetermined
2,2'-dihydroxybiphenyl	N					No Metabolism
benzophenone	N				N	No Metabolism
naphthol		G		T		Transformation, Weak Growth
1,2-dihydroxynaphthalene				N	N	No Metabolism
1,4-dihydroxynaphthalene		S		N		Solubilization
2,3-dihydroxynaphthalene				N		No Metabolism
2-naphthaleneethanol				N		No Metabolism
1,2-naphthoquinone		S		N		Solubilization
2-methyl-1,4-naphthoquinone				±S	N	Weak solubilization
2-methylanthraquinone		S		T		Transformation
9-anthraldehyde		S		T		Transformation
anthrone	N			±S	N	Weak solubilization
9-fluorenone				S		Solubilization
Aromatic acids						
benzoic acid	N				N	No Metabolism
4-methylbenzoic acid	N				G	Growth substrate
p-hydroxybenzoic acid	N				G	Growth substrate
3,4-dihydroxybenzoic acid	G				G	Growth substrate
salicylic acid	G				G	Growth substrate
4-methylsalicylic acid	N				N	No Metabolism
m–toluate	G				G	Growth substrate
protocatechuic acid					G	Growth substrate
gentisic acid					N	No Metabolism
o-phthalic acid					N	No Metabolism
2-naphthoic acid	N				N	No Metabolism

Table 3.5. (continued)

Pure compounds	Vapour plate	Spray plate	Overlay Plate	Liquid medium	Dissolved substrate	Interpretation of Results
Aromatic acids (continued)						
1-hydroxy-2-naphthoic acid				N	N	No Metabolism
2-hydroxy-3-naphthoic acid				N	N	No Metabolism
1-naphthylacetic acid				±S		Weak solubilization
2,6-naphthalenedicarboxylic acid				±S		Weak solubilization
Aromatic heterocycles and substituted aromatics						
quinoline				±S	N	Weak solubilization
isoquinoline				S		Solubilization
indole				±S	N	Weak solubilization
1,10-phenanthroline		T		N	N	Transformation
carbazole			N	±S	N	Weak solubilization
dibenzofuran				S		Solubilization
dibenzothiophene		T		T		Transformation
1-chloronaphthalene	N			N	N	No Metabolism
9-chloroanthracene				S		Solubilization
Aliphatic hydrocarbons and small organic acids						
n-pentane	G					Growth substrate
n-hexane	G					Growth substrate
hexanoic acid	G			G	N	Growth substrate
n-undecane	G					Growth substrate
n-dodecane	G					Growth substrate
n-hexadecane		G				Growth substrate
hexadecanoic acid	G			G	G	Growth substrate
n-heptadecane					N	No Metabolism
camphor	N				N	No Metabolism
sodium acetate					G	Growth substrate
succinic acid					N	No Metabolism

observed solublization of the test compounds was in fact due to ANT 17 and not a result of abiotic changes during shaking and incubation.

It is evident that ANT 17 possesses a broad substrate range for growth and transformation (Table 3.5), confirming observations on crude oil (Section 3.5.2). However the best growth of ANT 17 was qualitatively observed when phenanthrene was supplied. Phenanthrene, a three-ring PAH, was preferred over naphthalene, a two-ring PAH. This contradicts the general assumption that simpler compounds, i.e. with fewer rings, are more readily utilized and degraded than more complex compounds consisting of a greater number of rings (Cerniglia, 1992).

Benzene, the simplest aromatic compound, was inhibitory to ANT 17, most likely due to membrane damage as benzene is a toxic lipid solvent (Sikkema *et al.*, 1995). Growth on phenanthrene overlays was inhibited in the presence of 10, 50, and 100 µL of benzene supplied in the vapour phase.

ANT 17 was able to grow on many of the aromatic hydrocarbons and aromatic acids, while being only able to transform or solubilize some aromatic alcohols, aldehydes, ketones, quinones, heterocycles, and substituted aromatics. ANT 17 appears to prefer the di-aromatic hydrocarbons for growth with a variety of alkylated forms. Phenanthrene is the only tri-aromatic hydrocarbon tested capable of supporting growth. The remaining larger aromatic hydrocarbons were only transformed. Benzoic acid itself was not utilized, however a substitution in the *para* or fourth carbon of the aromatic ring of benzoic acid was suitable for growth.

Initially, the carbon utilization screens focussed on aromatic compounds, as ANT 17 had been isolated on 1-methylnaphthalene. Saturated compounds subsequently were tested to assess whether ANT 17 could biodegrade these compounds as well. ANT 17 grew on all saturated compounds tested, from pentane (*n*-C_5) to hexadecane (*n*-C_{16}) except heptadecane (*n*-C_{17}) which did not support growth. In addition, hexanoic and hexadecanoic acid supported growth. These results were surprising in view of the oil degradation studies, as no degradation of the saturate fraction was observed. This phenomenon was investigated further (Section 3.7.6).

Comparing these data to the hydrocarbon content of the isolation site of ANT 17 (Section 1.6.2 and Appendix A.3.1) several observations can be made. The JP8

contaminated soil contained mostly *n*-alkanes from *n*-C_9 to *n*-C_{14} with some mono- and di-aromatics. ANT 17 was found to grow on naphthalene, phenanthrene and fluorene, which are in higher concentration within the soil (200, 430, and 1000 ng/g respectively; Appendix A.3.1) compared to other aromatic hydrocarbons. The other larger aromatic compounds present in relatively small amounts were merely transformed by ANT 17. ANT 17 was also able to grow on *n*-alkanes from *n*-C_5 to *n*-C_{16}. This compares well to the *n*-alkane content of JP8 and the contaminated soil site. Based on the similarities between the compounds found within the JP8 contaminated soil of the isolation site and the qualitative screening results presented in Table 3.5, it seems reasonable that ANT 17 is able to grow on these compounds and survive in the Antarctic soil from which it came. It is likely that ANT 17 has been well adapted to degrade the compounds present within its isolation site, resulting in the broad substrate range of ANT 17.

The observation that ANT 17 grows on and degrades a wide variety of aromatic and saturated compounds implies that ANT 17 would be useful for the bioremediation of a site contaminated with diverse compounds such as are found in crude oil. Even though ANT 17 does not degrade or mineralize all compounds, it was be a useful organism at a contaminated site as perhaps it could initiate the biodegradation process allowing other organisms to then complete the process.

3.6.2. Comparison to known pathways

Since the best compound for supporting ANT 17 growth was phenanthrene, a comparison of the data presented in Table 3.5 to the known phenanthrene degradation pathways would seem appropriate. The phenanthrene biodegradation pathways are presented in Figure 1.7 of Section 1.3.2. ANT 17 did not grow on 1-hydroxy-2-naphthoic acid, which is the compound at the branch point of both pathways for complete phenanthrene degradation. Similarly, ANT 17 did not grow on *o*-phthalic acid or 1,2-dihydroxynaphthalene, which are both intermediates of the two possible phenanthrene pathways. ANT 17 did grow on protocatechuic acid or salicylic acid, which are the end points of the phenanthrene pathways illustrated prior to mineralization.

The observation that ANT 17 does not grow on the intermediates but grows on the end products of the known pathways for phenanthrene biodegradation is interesting.

Perhaps ANT 17 utilized a different mechanism to reach the same end products of phenanthrene biodegradation, which would suggest that this organism possesses a unique pathway. Alternatively, the intermediates may not have supported ANT 17 growth or transformation due to physical limitations. These could result from lack of transport mechanisms for the intermediates into the cell, or a lack of induction for enzyme synthesis by these intermediates. It is obvious that more research is required in this area, including the determination of the phenanthrene pathway utilized by this organism as well as the regulation and induction of the genes encoding the enzymes of this pathway. This could also be extended to other pathways.

When ANT 17 was grown on fluoranthene, brilliant orange coloured transformation products were observed after a few days, which subsequently disappeared leaving the surrounding medium a light beige colour. These results directly paralleled the observations made by Mueller *et al.* (1990). Their study with *S. paucimobilis* EPA505 demonstrated the appearance of a reddish-orange metabolite of fluoranthene and its subsequent disappearance to a light brown colour. The appearance of the reddish-orange colour coincided with the appearance of two metabolites. When the light brown colour was present, a further accumulation of the second metabolite was observed, with the complete disappearance of the first metabolite and fluoranthene. These metabolites were not identified. It would be interesting to compare ANT 17 directly to strain EPA505 in order to determine if they produce the same metabolites and to determine what these metabolites are.

3.7. Quantification of ANT 17 growth on various compounds

This portion of the thesis was conducted to quantify the growth of ANT 17 on various compounds that supported growth or transformation, based on the qualitative results presented in Section 3.6.

3.7.1. Utilizing the BCA assay for protein determination

The BCA assay for protein determination was utilized as described in Section 2.9. Early assays in the trial stage were conducted with various C sources added to Mineral medium (Appendix B.3). Unusually high absorbance readings outside the range of the

calibration curve were occurring in all samples including controls. It was determined that the salt stock added to the Mineral medium was causing interference in the assay. At this time Mueller Mineral salts medium was tested (Mueller *et al.*, 1989; Appendix B.3) as this medium was utilized for BCA assays by this research group. This medium did not interfere with the assay. Although the qualitative compound screens were conducted with Mineral medium, ANT 17 was found to grow on and transform the same compounds selected for testing with Mueller Mineral salts medium. Since these qualitative results were similar, the Mueller Mineral salts medium was selected for quantitative growth studies of ANT 17 on select compounds.

3.7.2. Growth in the presence and absence of Tween® 80

Studies by Mueller *et al.* (1990) describe a *Sphingomonas* (formerly *Pseudomonas*) *paucimobilis* isolate EPA505 that was capable of utilizing fluoranthene. These experiments included Tween® 80 to increase the bioavailability of the aromatic hydrocarbon for biodegradation. *S. paucimobilis* isolate EPA505 was not capable of utilizing Tween® 80.

ANT 17 was tested for its ability to grow in the absence and presence of Tween® 80 utilizing Mueller Mineral Salts medium with 0.2% w/v glucose. Tween® 80 was added as described in Section 2.1.3. Cultures were incubated at 22°C and the OD_{600} and viable counts (PCA plate counts) were conducted at time zero and after 18, 40, and 63 h. After 63 h the OD_{600} values were 0.48 and 0.32 in the absence and presence of Tween® 80 respectively, while the viable counts were 2.4×10^8 and 2.2×10^8 CFU/mL, respectively. ANT 17 growth was not negatively affected by Tween® 80 and was therefore suitable for further tests with hydrocarbon substrates.

ANT 17 growth on phenanthrene and fluoranthene was assessed as described in Section 2.1.3. ANT 17 was inoculated from a 10 mM phosphate buffer suspension of cells obtained from a NAG plate. Flasks containing no C source, Tween® 80 only, phenanthrene added "dry" with and without Tween® 80, phenanthrene added "wet" with Tween® 80, and fluoranthene added "dry" with Tween® 80 were incubated at 30°C in

the dark with shaking. An uninoculated control was also included (containing phenanthrene added "dry" with Tween® 80).

Adding phenanthrene "dry" with Tween® 80 produced better growth than adding the phenanthrene "wet" with Tween® 80 (Figure 3.20). In addition the inclusion of Tween® 80 greatly increased the solubility and bioavailability of phenanthrene, as seen by enhanced growth. ANT 17 did not grow on fluoranthene, confirming the results presented in Table 3.5. ANT 17 in the culture containing only Tween® 80 grew to the same extent as fluoranthene, which was above the abiotic and no carbon controls. It appears that ANT 17 was utilizing Tween® 80 as a carbon source. ANT 17 did not appear to utilize Tween® 80 during the previous trial utilizing glucose as a C source. The apparent utilization of Tween® 80 in this analysis was not surprising, as all of the *S. paucimobilis* isolates studied by Yabuuchi *et al* (1990) had the ability to utilize Tween® 80. ANT 17 most likely did not utilize Tween® 80 in the previous analysis in the presence of glucose due to diauxic growth effects. In this case with Tween® 80 as the only C source, it was utilized.

In a second experiment, growth of ANT 17 was assessed on phenanthrene, fluoranthene, pyrene, chrysene, and anthracene. All compounds were added to flasks in stock solutions as described in Section 2.1.3 and the solvent allowed to evaporate (added "dry") prior to the addition of sterile medium and inoculation. Tween® 80 was included in all flasks. Abiotic, no C, and no C with Tween® 80 control flasks were included. ANT 17 was inoculated from a cell suspension in 10 mM phosphate buffer from cells obtained from a NAG plate. The flasks were incubated at 30°C with shaking in the dark and growth was measured as protein concentration.

The results presented in Figure 3.21 show that again phenanthrene was a growth substrate while the other aromatic hydrocarbons were not: protein concentrations were not significantly above the protein concentration of the no C with Tween® 80 control. Tween® 80 was serving as a C source since the protein concentration in this culture was greater than the abiotic and no C control values. Since Tween® 80 was being utilized

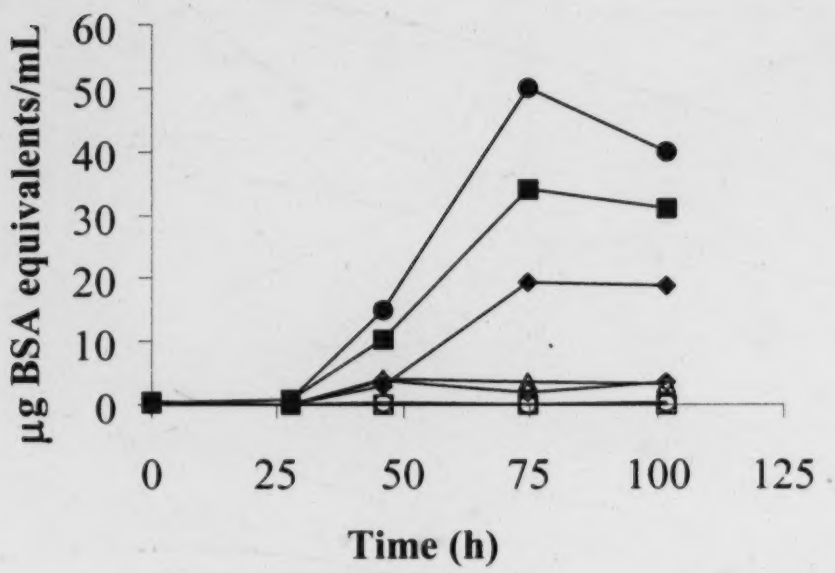

Figure 3.20. Growth of ANT 17 at 30°C in Mueller Mineral salts medium. Abiotic control (○); ANT 17 cultures with no C source (□), with Tween® 80 (△), with phenanthrene added "dry" (◆), with phenanthrene added "dry" with Tween® 80 (●), with phenanthrene added "wet" with Tween® 80 (■), and with fluoranthene added "dry" with Tween® 80 (◇).

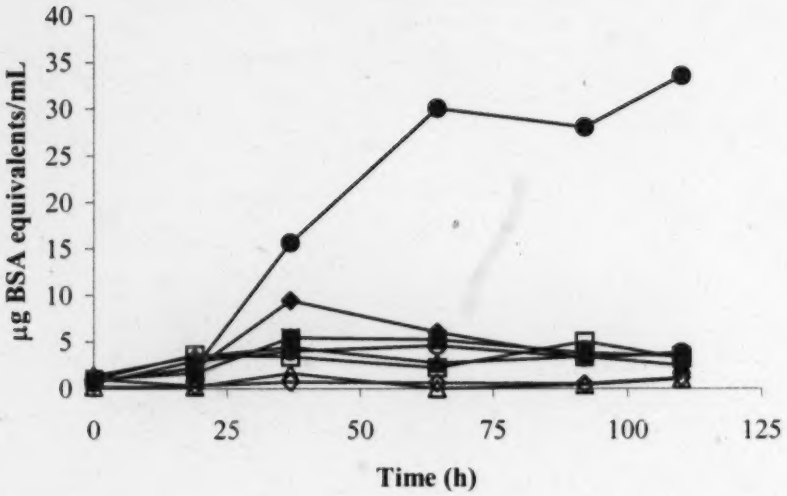

Figure 3.21. Growth of ANT 17 at 30°C in Mueller Mineral salts medium. Abiotic control (△); ANT 17 cultures with no C source (◇), with Tween® 80 (□), and with aromatic hydrocarbons added "dry" with Tween® 80: phenanthrene (●), fluoranthene (■), pyrene (◆), chrysene (▲), and anthracene (○).

as a C source, in further studies it was not used as a detergent to increase the solubility of aromatic hydrocarbons.

Triton X-100 is another common surfactant utilized in biodegradation studies to increase the bioavailability of substrates with low solubility and its effect on ANT 17 growth was tested. Triton X-100 was inhibitory to ANT 17 growth, and culturability of ANT 17 after exposure to 0.1% solution of this detergent was nil. Similar results were obtained with *S. paucimobilis* EPA505 (Willumsen and Karlson, 1998) where mineralization of glucose and fluoranthene and culturability were inhibited by the presence of Triton X-100 in mineral medium. However, in the presence of Ca^{2+}, Triton X-100 doubled the rate of fluoranthene mineralization while reducing the culturability by only 10%. Triton X-100 adversely affected the cytoplasmic membrane. Isolate EPA505 had previously tolerated up to 2% Triton X-100, with enhanced fluoranthene mineralization, but only when grown in Bushnell-Haas medium (Lantz *et al.*, 1995).

3.7.3. Growth dependence on inoculum density

3.7.3.1. Preliminary observations

Based upon the growth curve data obtained from the temperature studies (Section 3.3.2) and from other observations, it was noted that ANT 17 exhibits an unusually long lag time until a critical cell density was reached, at which time rapid log growth is initiated. The actual "point" or critical cell density point was not known. This phenomenon was investigated briefly to determine if ANT 17 was undergoing some sort of quorum-sensing effect. In general, quorum sensing, or autoinduction, in Gram-negative bacteria, is the result of an excreted acylhomoserine lactone signal within a bacterial population. This signal monitors cell density in order to achieve a desired result via gene activation, such as colonization of a host for opportunistic pathogens when cell numbers are sufficiently high (Greenberg, 1997).

The first attempt to understand these observations utilized a log phase grown ANT 17 culture in TSB. This was used as an inoculum source for further TSB cultures containing sterile dialysis tubing with 3 mL of the grown ANT 17 culture, supernatant from the grown ANT 17 culture, grown *E. coli* culture, or sterile TSB medium. The OD_{600} of the cultures was followed during incubation at 22°C, in order to determine if the

presence of some diffusible factor from either the grown ANT 17 culture or the supernatant would initiate rapid log growth of ANT 17 compared to the sterile medium and the *E. coli* culture. This method proved to be too cumbersome for accurate results.

The next approach tried was to add various volumes (0%, 10% and 30%) of filter sterilized ANT 17 culture supernatant to lag phase ANT 17 cultures in TSB. If a cellular excreted "factor" present in the supernatant was required for cell density signaling to initiate rapid log growth, optical density readings might have detected the decreased lag time. The final volumes of all cultures was maintained at 200 mL TSB by accounting for the volume and nutrients added in the form of the filter sterilized supernatant. The OD_{600} readings after 44 h showed that the cultures receiving either 0% or 10% supernatant (OD_{600} of 3.40 and 3.36 respectively) grew better than the culture receiving 30% (OD_{600} 2.67). This may have been due to the presence of toxic metabolic products in the filter sterilized supernatant, which inhibited growth.

In order to investigate the effect of inoculum density on ANT 17 growth rates and lag times another experiment was conducted. ANT 17 was grown in QTSB buffered to pH 7.65, incubated at 22°C. During the late lag phase (at 105 h), 100 µL of this culture was used to inoculate two QTSB cultures buffered to pH 6.0 and 7.5, respectively. During the late log phase (at 166 h) of the original QTSB culture, 100 µL was used to inoculate another two QTSB cultures also buffered to pH 6.0 and 7.5, respectively. The OD_{600} of all cultures was monitored. Figure 3.22 illustrates the results. Little difference, other than lag times occurred in the growth rates and generation times of the cultures once logarithmic growth was achieved. At the more optimal pH of 6.0, there was little difference in the lag time between cultures inoculated with lag phase or log phase cultures. In contrast, at pH 7.5, inoculation with a higher density inoculum dramatically reduced the lag time. Since the growth rates and generation times were not affected by either inoculum density and the only difference was the lag time, subsequent experiments were designed to employ a high inoculum density.

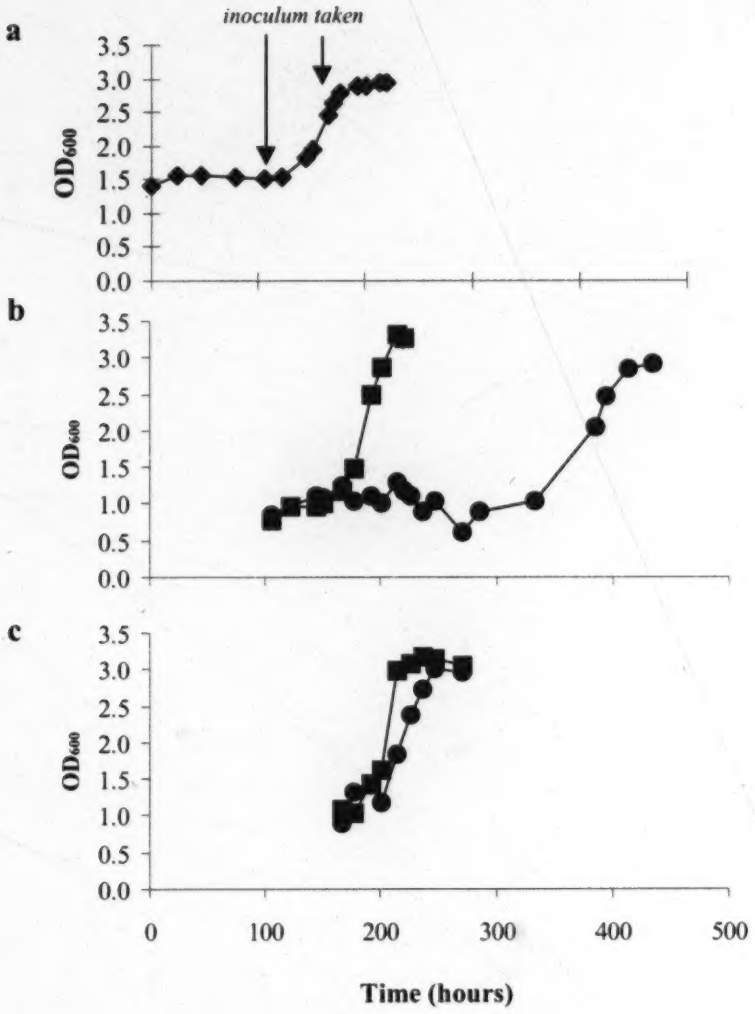

Figure 3.22. Growth of ANT 17 at 22°C in QTSB. **a.** QTSB culture buffered to pH 7.65 (◆) was used as an inoculum source at 105 h (lag phase) and at 166 h (log phase). **b.** ANT 17 cultures inoculated from lag phase cells into QTSB buffered to pH 6.0 (■) and pH 7.5 (●). **c.** ANT 17 cultures inoculated from log phase cells into QTSB buffered to pH 6.0 (■) and pH 7.5 (●).

3.7.3.2. Comparison to other bacterial species

The effect on ANT 17 growth of differing inoculum densities was compared *P. fluorescens* LP6a, and *E. coli*. Each organism was grown on PCA. A cell suspension in 10 mM phosphate buffer was prepared by harvesting colonies from each plate with a sterile glass spreader. Each cell suspension was then used to inoculate tubes containing 10 mL TSB buffered to pH 6.6 or 7.4 at 0.1%, 0.3%, 1%, or 3% v/v (10 µL, 30 µL, 100 µL, or 300 µL, respectively). These tubes were incubated on a tube roller at 28°C. OD_{600} measurements were obtained at time zero and after 24 and 48 h (Figure 3.23). ANT 17 appears to be affected by the different inoculum densities used, whereas *E. coli* and LP6a were not. It is likely that the observed inoculum density effects are a result of differing growth rates under the various conditions.

3.7.3.3. Growth on various PAHs

To determine the effect of increasing the inoculum density on ANT 17 growth on PAHs, another experiment was conducted utilizing the BCA assay for protein determination. Mueller Mineral salts medium (5 mL tube cultures) was amended with phenanthrene, pyrene, chrysene, anthracene, and fluoranthene at a final concentration of 5 mmol/L. Comparisons were made to uninoculated, no C, and no C + DMF controls (DMF was the solvent used to add the PAH source). ANT 17 was grown in TSB at 22°C, harvested and suspended in 10 mM phosphate buffer to prepare inocula having OD_{600} values from 0.2 to 2.2. One hundred microlitres of each inoculum was added to the various culture tubes and incubated at 28°C for 48 h. Samples for protein concentration determination were obtained at initial and final times. The results are presented in Figure 3.24 (at time zero only phenanthrene shown, as it represents all of the time zero protein concentrations for each PAH tested).

The no C and no C + DMF controls show an approximate doubling in protein concentration. This would represent one doubling of the cells inoculated before growth was arrested due to a lack of C and energy source. No additional growth occurred with the solvent used to add the concentrated PAH stocks to the medium, as the no C +

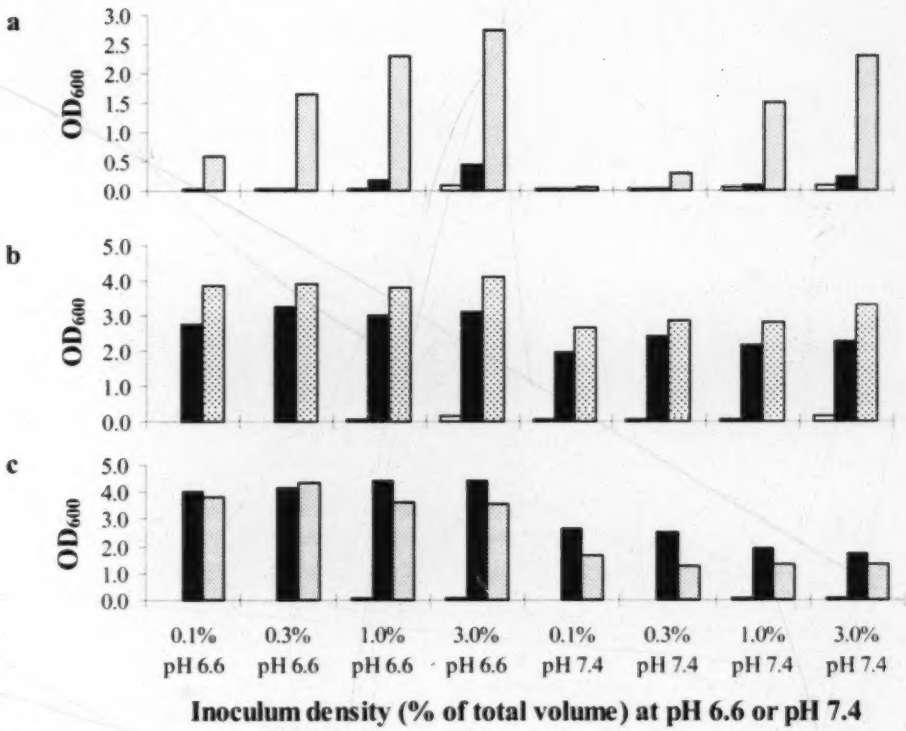

Figure 3.23. Growth of ANT 17 (**a**), LP6a (**b**), and *E. coli* (**c**) at inoculum densities from 0.1% to 3%, in TSB buffered to pH 6.6 or 7.4. OD_{600} measured at time zero (open box), 24 h (filled box) and 48 h (stippled box).

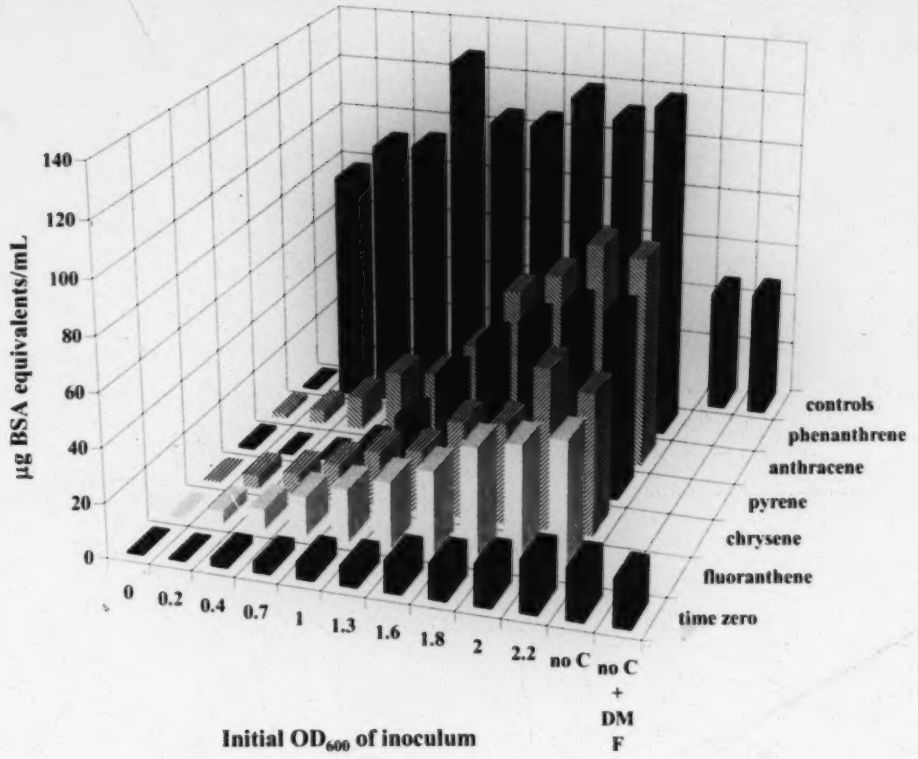

Figure 3.24. ANT 17 cultures grown on various PAHs starting with different inoculum densities (OD_{600} from 0.2 to 2.2). Mueller Mineral salts medium, incubated at 28°C for 48 h, with 5 mmol/L phenanthrene (solid black), anthracene (downward right cross hatch), pyrene (fine dotted), chrysene (downward left crosshatch) and fluoranthene (white). No carbon (no C) and no carbon plus DMF solvent (no C + DMF) controls shown at time zero and time final (large dotted). Time zero series (large dotted) is representative of each inoculum density for all PAHs. Uninoculated controls represented by an initial OD_{600} of inoculum at 0.0.

DMF values were nearly identical to the no C values. The uninoculated controls show no net increase in protein, as expected. The results for phenanthrene after 48 h indicate that ANT 17 was able to utilize this PAH as a C and energy source as expected from previous qualitative results (Table 3.5). Growth was extensive regardless of the initial inoculum density.

For anthracene, a decrease in insoluble substrate was observed (visually and non-quantitatively) in all inoculated cultures compared to the uninoculated control. All inoculum densities above OD_{600} 1.3 resulted in protein increases greater than the no C controls. The results for ANT 17 growth on pyrene were similar, as physical changes were observed with the insoluble substrate within inoculated cultures. Similarly, inoculum densities above OD_{600} 1.3 resulted in a net increase in protein concentration that was more than double the original amount present in time zero and no C controls.

The chrysene cultures also exhibited a decrease in insoluble substrate, however, protein did not increase significantly in any of the cultures. Fluoranthene did not serve as a growth substrate for ANT 17 either, based on the lack of increase in protein concentration. However, ANT 17 did transform fluoranthene to a vivid orange coloured product(s) that increased in colour and intensity with increasing inoculum density.

Based on the results of this experiment, the cultures with lower inoculum densities may have still been in lag phase, prior to possibly achieving the same overall culture maximum. To clarify whether increasing the inoculum density increased the overall cellular yield or biodegradation on these PAH carbon sources, the cultures would require a longer incubation time period, and sampled more frequently. In short, further studies are required to elucidate the effect of inoculum density protocols on growth of ANT 17 and biodegradation. A study by Ye *et al.* (1996) with *S. paucimobilis* EPA505 noted that an increase in the rate and extent of benz[a]anthracene degradation with increasing cell densities.

3.7.4. Dependence on PAH concentration

Low bioavailability of a compound or potential substrate is often viewed as a limiting factor for its degradation and removal (Leahy and Colwell, 1990; Atlas and Bartha, 1992; Cerniglia, 1992). The effect of increasing concentrations of phenanthrene

on ANT 17 growth was assessed. ANT 17 was harvested from a TSB culture grown at 22°C and suspended in 10 mM phosphate buffer to an OD_{600} of 2.0. One hundred microlitres of this cell suspension was inoculated to 5 mL Mueller Mineral salts medium in test tubes. Phenanthrene was added from a concentrated DMF stock solution to achieve final concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, or 2.5 mmol/L. No C and no C + DMF controls were included to account for cell growth in the absence of a C source. An uninoculated control containing 2.5 mmol/L phenanthrene was also included. The experiment was conducted twice: one trial was incubated at 22°C for 14 days and the second independent trial incubated at 28°C for 7 days.

Time zero and time final samples were analyzed for protein. The assay procedure required a slight modification to account for the differences in insoluble phenanthrene present, due to the differing concentrations used in these experiments. After the cell lysis incubation at 60°C for 30 min, 100 µL DCM was added to each microfuge tube, briefly vortexed and pulsed in a microfuge to separate the phases. The aqueous phase (containing the cell lysate) was removed and incubated with the working reagent for the assay as described previously. This modification removed the insoluble substrate while minimizing loss of protein. Preliminary trials with this method indicated that this was sufficiently accurate to proceed.

The results of both trials are presented in Figure 3.25. It appears that low phenanthrene concentrations (0.01 to 0.05 mmol/L at 22°C and 0.01 to 0.1 mmol/L at 28°C) were not sufficient for ANT 17 growth above the no C controls, as there is a lack in proportionality of protein increase with respect to increase in phenanthrene. The higher concentrations of phenanthrene gave the best protein yield of ANT 17 and appear more proportional to increasing concentrations of phenanthrene. The protein yield at 28° is approximately double the protein yield at 22°C. Given that the 22°C were incubated for 14 days, as opposed to the 28°C cultures incubated for 7 days, it is likely cell lysis had occurred in the 22°C cultures due to the longer incubation time.

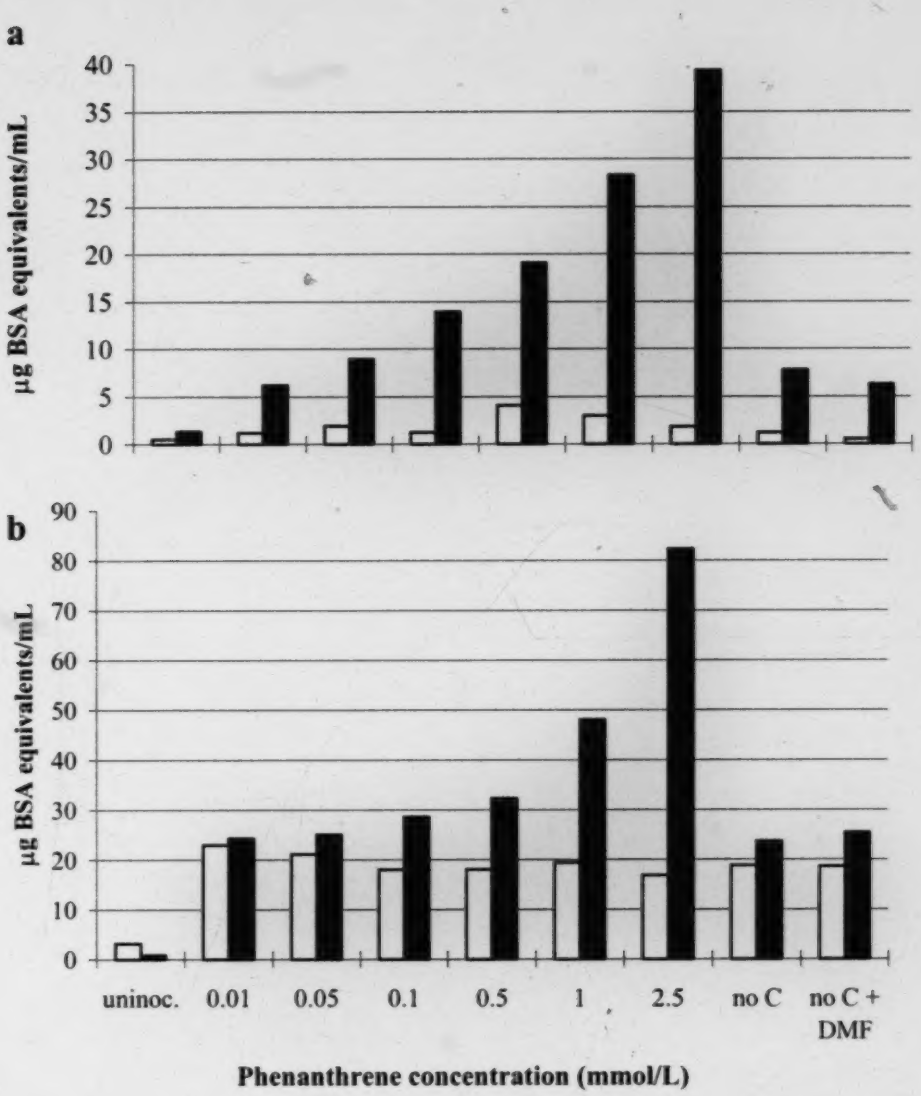

Figure 3.25. Growth of ANT 17 on various concentrations of phenanthrene in Mueller Mineral salts medium at 22° (**a**), and 28°C (**b**) for 14 and 7 days, respectively. Time zero (open bar) and time final (solid bar). Growth compared to uninoculated (uninoc.), no C, and no C + DMF controls.

The solubility of phenanthrene is 1.3 mg/L (Cerniglia, 1992). The concentrations used in this study were all above the solubility limit as 0.01 mmol/L corresponds to 1.8 mg/L. It is likely that growth of ANT 17 is not limited by low phenanthrene concentration *per se*, rather it is most likely limited by the rate of dissolution. Increasing the amount of phenanthrene well above the solubility limit may allow more dissolution of phenanthrene as solubilized molecules are utilized and degraded. It should be noted that the concentrations of phenanthrene used in this study are greater than those normally found in the environment. At the isolation site of ANT 17, phenanthrene was present at 430 ng/g soil (Appendix A.3.1), well below the solubility limit for phenanthrene. Bioavailability of this PAH in the environment would be further reduced by phenomena such as adsorption to soil particles. Further studies would need to be conducted to determine the limits of bioavailability of PAHs for ANT 17 degradation.

3.7.5. Growth on aromatic compounds

Growth of ANT 17 on various aromatic compounds was assessed by protein determination via the BCA assay. Inoculum was prepared from a washed TSB culture pellet grown at 22°C. Phenanthrene, pyrene, chrysene, anthracene, fluoranthene, fluorene, naphthalene, dibenzothiophene, triphenylene and salicylic acid were added to Mueller Mineral salts medium from concentrated DMF stocks to achieve a final concentration of 2.5 mmol/L. *m*-Xylene was added directly to the liquid medium at this concentration. A sterile control containing phenanthrene and a no C control were also included. Incubation proceeded at 22°C with sub-samples taken at various times for protein determination. Figure 3.26 illustrates the results obtained. Growth on phenanthrene and naphthalene was apparent, whereas growth on the remaining aromatic compounds tested was not above that of the no C control. Coloured products were noted in the fluoranthene, fluorene, and dibenzothiophene cultures. Although ANT 17 was transforming these compounds, it was not utilizing them as a C and energy source. Changes in the solubility of the insoluble substrate were visually noted in the other cultures, which also indicated transformation or solublization.

Although ANT 17 cannot grow on many of these compounds tested, their transformation *in situ* may positively contribute to the ultimate removal of these

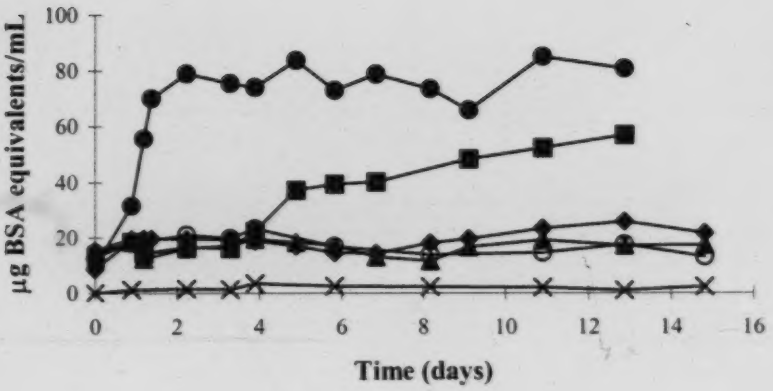

Figure 3.26. Growth of ANT 17 at 22°C in Mueller Mineral salts medium with various aromatic compound carbon sources. Sterile control (×), no C control (○), phenanthrene (●), naphthalene (■), dibenzothiophene (◆), and fluoranthene (▲). Results for salicylic acid, *m*-xylene, fluorene, triphenylene, pyrene, chrysene and anthracene were not presented as they closely resembled values for the no C control.

petroleum contaminants. Through cometabolism, ANT 17 may initiate biodegradation by solubilizing or oxidizing recalcitrant compounds, allowing other organisms to complete the biodegradation process. The next course of research would be to determine how ANT 17 contributes to the removal of various petroleum related compounds as a member of a consortium. Such studies could be carried out simply in liquid shake flask cultures or more elegantly within soil microcosms.

3.7.6. Growth on saturated compounds

Despite the fact that ANT 17 grew on various *n*-alkanes and carboxylic acids during qualitative screening (Table 3.5, Section 3.6.1) it did not alter the saturate profile of PBCO (Figure 3.11, Section 3.5.1). To determine if growth on the saturated fraction was dependent upon the absence of the aromatic fraction (i.e. a catabolite repression effect) ANT 17 was incubated at 22°C on a Mineral medium plate containing PBCO saturated fraction spread onto the surface. A saturate-degrading positive control bacterium was also assessed. Growth of the saturate degrader occurred, whereas ANT 17 did not grow when PBCO saturates were provided as the sole C and energy source.

An attempt to grow ANT 17 on 200 µL of the PBCO saturated fraction alone in liquid Mineral medium, amended with N,P nutrient amendment, was also conducted. A sterile control and inoculated culture were incubated at 28°C for 1 week, extracted, and analyzed by GC. The two saturate profiles obtained were identical and paralleled the results presented in Figure 3.11. That is, ANT 17 did not appear to grow on the saturated fraction of PBCO either as part of the complete PBCO mixture or alone. Perhaps the complete saturated fraction was toxic to ANT 17, whereas individual compounds tested were suitable as growth substrates.

Growth of ANT 17 on individual saturated compounds was next assessed and quantified utilizing the BCA assay for protein concentration. *n*-Alkanes (*n*-C_5, *n*-C_6, *n*-C_{11}, *n*-C_{12}, *n*-C_{16}, *n*-C_{17}), hexanoic acid, hexadecanoic acid, glucose and sodium acetate were individually provided as the sole C source at a final concentration of 1 mmol/L in Mueller Mineral salts medium as described in Section 2.1.3. ANT 17 was inoculated from a QTSB culture and incubated at 28°C, and growth was compared to sterile and no C controls. Propyl alcohol was also tested as a C source, as hexadecanoic acid was

dissolved in this solvent as a concentrated stock. Glucose and sodium acetate were included to illustrate the potential maximum protein yield under these conditions. Figure 3.27 illustrates the results. The *n*-alkane results were equivalent to the no C control, and are omitted from the figure for clarity. Growth on glucose was significantly greater than growth on sodium acetate, while growth on hexanoic acid was similar to glucose. Growth on hexadecanoic acid was greater than glucose, however, the propyl alcohol alone also supported ANT 17 growth, and likely contributed to the total protein detected.

The lack of growth on the *n*-alkanes was surprising as ANT 17 did grow on all of these saturated compounds, except heptadecane, when supplied to ANT 17 in or on solid medium (Table 3.5). Perhaps ANT 17 requires physical contact with either the compound directly or its vapour. Neoprene stoppers were employed to seal the culture flasks during incubation to minimize the loss of these substrates during incubation, and it is possible that some of the substrates were lost when the flasks were opened during sampling. The vapour pressure in the liquid cultures may not have been sufficient for growth on these substrates. In addition, these liquid cultures were incubated at 28°, as opposed to 22°C at which the plates were incubated for the data presented in Table 3.5. The rapid growth of ANT 17 on the carboxylic acids was not surprising as these compounds are generally more readily degraded than the parent *n*-alkanes, as discussed in Section 1.3.3. This is due to the fact that the initial oxidation reactions of the *n*-alkane have been completed and the organism can utilize the carboxylic acid directly via β-oxidation.

3.8. Mineralization of ^{14}C-radiolabelled compounds

After determining that ANT 17 could utilize and transform a wide variety of petroleum associated compounds, the next course of research was to determine if ANT 17 could completely biodegrade (mineralize) various compounds to CO_2. This is important to determine the fate of petroleum associated compounds through ANT 17 biodegradation. Such knowledge would be required to understand the fate of these compounds in the environment prior to any *in situ* applications of ANT 17. This section describes those studies.

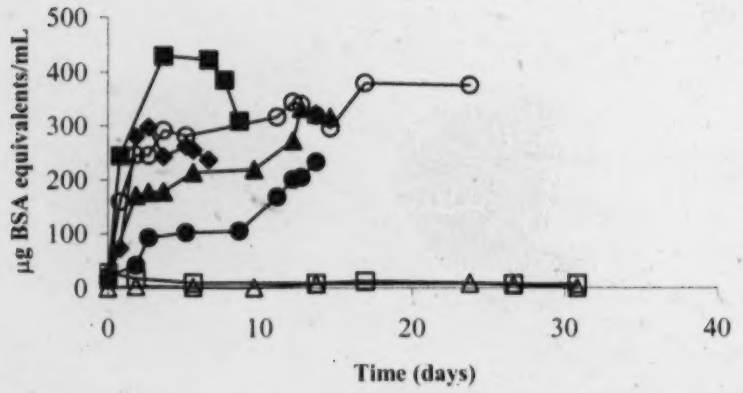

Figure 3.27. Growth of ANT 17 at 28°C in Mueller Mineral salts medium with various carbon sources. Sterile control (△), no C control (□), glucose (○), sodium acetate (●), propyl alcohol (▲), hexanoic acid (◆), and hexadecanoic acid (■).

3.8.1. Preliminary trials and optimization of conditions

In early trials of the protocol for mineralization studies, mineralization of glucose and phenanthrene was assessed and compared to uninoculated controls. ANT 17 was harvested from a QTSB culture and suspended in 10 mM phosphate buffer to be used as an inoculum. Mineral medium was utilized with 1 mmol/L of glucose or phenanthrene (described in Section 2.11). The cultures and controls were incubated at 22°C in the dark, sampled at various times, flushed and enumerated for $^{14}CO_2$ as detailed in Section 2.11. By 45 h, ANT 17 had mineralized glucose to 39.4% $^{14}CO_2$. Phenanthrene was 69.5% mineralized by 125 h.

Next, phenanthrene mineralization was assessed under various limiting conditions to compare these results to the oil degradation studies (Section 3.5). At 22°C, ANT 17 was grown in triplicate on mineral medium with 1 mmol/L of phenanthrene under static and shaking conditions without N,P nutrient amendment; and with shaking plus N,P; NO_3^-,P; or NH_4^+,P. An 8°C shaking culture lacking N,P was also included. All results were compared to a sterile control (shaking). Figure 3.28 presents the results obtained. While the culture was in lag phase, no significant deviations were observed, however, once mineralization commenced it was noted that often one or two of the triplicate cultures would be slightly ahead of the other culture(s), which increased the observed standard deviations.

ANT 17 readily mineralized phenanthrene as expected, as this PAH had previously been determined to support extensive growth. Mineralization at 8°C was also expected as degradation of the various oils tested (Section 3.5) was previously observed at cold temperatures. However, the lack of mineralization in the presence of the various nutrient amendments was not expected. Generally (as discussed in Sections 1.3.3 and 1.5), biodegradation and mineralization are enhanced with nutrient amendments when nutrients are limiting such as unsupplemented in mineral medium. Phenanthrene mineralization with nutrient amendments was expected to parallel mineralization in the absence of nutrients. The oil degradation results in Section 3.5 indicate that biodegradation of the various oils and the refined product tested were only marginally enhanced, if enhanced at all by the inclusion of nutrients. Degradation was not inhibited

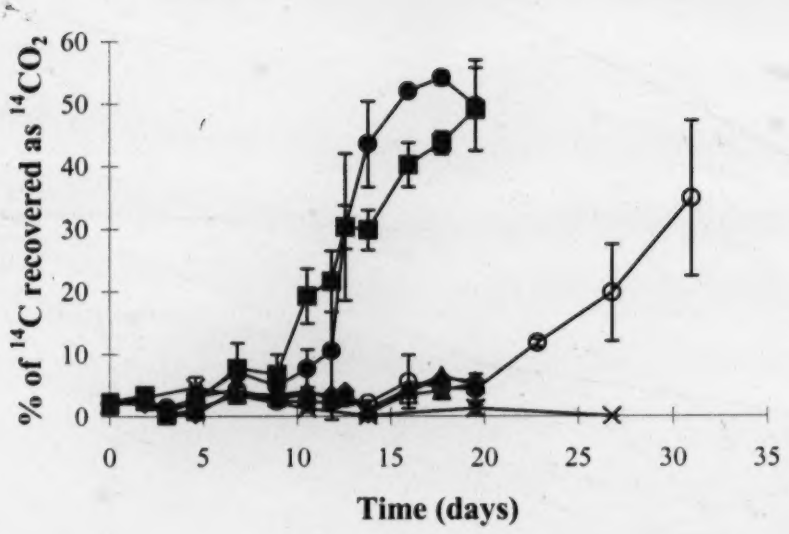

Figure 3.28. ANT 17 mineralization of [9-^{14}C]-phenanthrene in Mineral medium under various conditions. Sterile control (×), at 22°C static and without N,P (■), shaking and without N,P (●), with N,P (▲), with NO_3^-,P (◇), with NH_4^+,P (△), and at 8°C shaking without N,P (○). All conditions tested in triplicate. Error bars shown for cultures lacking nutrient amendments and sterile control and indicate one standard deviation.

by the inclusion of nutrients as seen in this analysis. Without conducting further research, it is unknown why this occurred. The extensive mineralization of phenanthrene with and without shaking directly compares with results presented in Section 3.5.3, where no difference in oil degradation of PBCO aromatic fraction was observed between the shaking and static cultures. It would again appear that enhanced aeration or agitation is not a limiting factor for biodegradation.

To determine if the conditions for mineralization could be optimized further, phenanthrene mineralization was assessed utilizing Mineral medium compared to a slightly modified Mineral medium as described in Section 2.11. The Mineral medium was modified to resemble the phenanthrene overlay medium (Appendix B.3) at the pH optimum for ANT 17 (Section 3.4.2). In addition, two concentrations of phenanthrene were tested (1 mmol and 2.5 mmol/L) while maintaining a constant amount of radiolabel in the cultures. Mineralization was compared to a sterile control. Incubation proceeded at 28°C with shaking for all cultures. These cultures were prepared as single flasks rather than triplicates, as this experiment was intended to serve as an optimization trial. Figure 3.29 illustrates the results.

From the results, it appears that by providing a small amount of yeast extract (0.01 % w/v) in the medium and by buffering to the optimum pH of 6.4, the lag time prior to rapid mineralization of phenanthrene can be reduced from 30 days to 15 days. The extent of mineralization was unchanged for both types of medium. Without individually testing the effect of adding yeast extract or of buffering the medium, the effect of these modifications on reducing lag time remains undetermined. The addition of a higher concentration of phenanthrene did not greatly change the overall mineralization of phenanthrene and was more cumbersome to sample from as the increased insoluble substrate would frequently plug the sampling needles. Based on these results, it was decided to utilize the modified medium to decrease lag time, and to maintain the concentration at 1 mmol/L for ease of sampling.

3.8.2. Mineralization and distribution of radiolabel

To determine the potential of ANT 17 to mineralize other petroleum-related compounds, an experiment was conducted utilizing the Modified Mineral medium

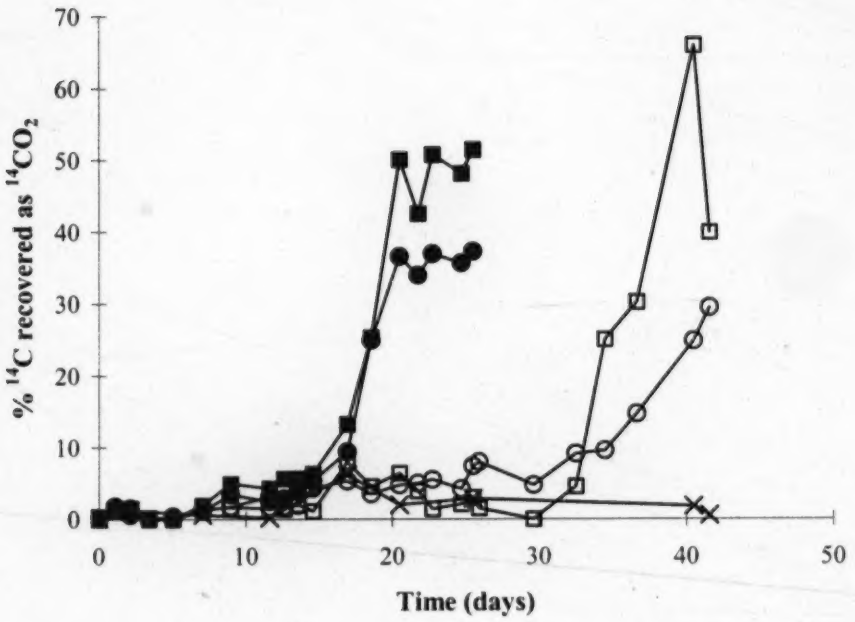

Figure 3.29. ANT 17 mineralization of [9-^{14}C]-phenanthrene at 28°C in Mineral medium and Modified Mineral medium. Conditions varied as follows: phenanthrene at 1 mmol/L in unamended Mineral medium at pH 7.0 (□) and Mineral medium + 0.01% yeast extract with buffering to pH 6.4 (■), phenanthrene at 2.5 mmol/L in unamended Mineral medium at pH 7.0 (○) and Mineral medium + 0.01% yeast extract with buffering to pH 6.4 (●), sterile control (×).

described in Section 3.8.1. Radiolabelled glucose, hexadecane, palmitic acid, phenanthrene, fluoranthene or pyrene were added as described in Sections 2.7 and 2.11. ANT 17 was harvested from a QTSB culture, suspended in 10 mM phosphate buffer and used as the inoculum. Each of the radiolabelled C sources was supplied at a final concentration of 1 mmol/L with approximately 500 000 dpm per culture flask to ensure sufficient radiolabel for completion of the radiolabel distribution portion of the experiment (as described in Section 2.11). Incubation proceeded with shaking at 28°C. A series of phenanthrene flasks was also incubated at 1° and 8°C with shaking as well as at 28°C without shaking. All inoculated cultures were prepared in triplicate for each test condition, in addition to the inclusion of a single uninoculated sterile control.

Figure 3.30 displays the results obtained from the $^{14}CO_2$ recovery time course. The sterile control values are not plotted as they never exceeded 1% $^{14}CO_2$. ANT 17 mineralized 37% of glucose within 3 days incubation. This confirmed previous results described in Section 3.8.1 where approximately 40% mineralization of glucose had occurred by 2 days. Error bars (representing one standard deviation) in Figure 3.30 indicate good reproducibility among the triplicate cultures incubated with glucose as the sole C source.

ANT 17 mineralized 30% palmitic acid by 7 days but did not mineralize hexadecane within 48 days incubation. This confirms previous growth results discussed in Section 3.7.6 and Figure 3.27 where an increase in protein was observed with ANT 17 grown on palmitic acid but not hexadecane. Error bars are shown for the cultures with palmitic acid, and indicate good reproducibility among the triplicates. Error bars are omitted from the hexadecane data series, as standard deviations were less than 1% because mineralization did not occur.

ANT 17 did not mineralize fluoranthene or pyrene confirming, the lack of growth (measured as protein) described in Section 3.7.5 and Figure 3.26. An orange coloured transformation product(s) appeared in the fluoranthene cultures by 3 days and disappeared by 10 days, consistent with previous observations. Error bars were omitted from these series in Figure 3.30 as mineralization did not occur and standard deviations were less than 1%.

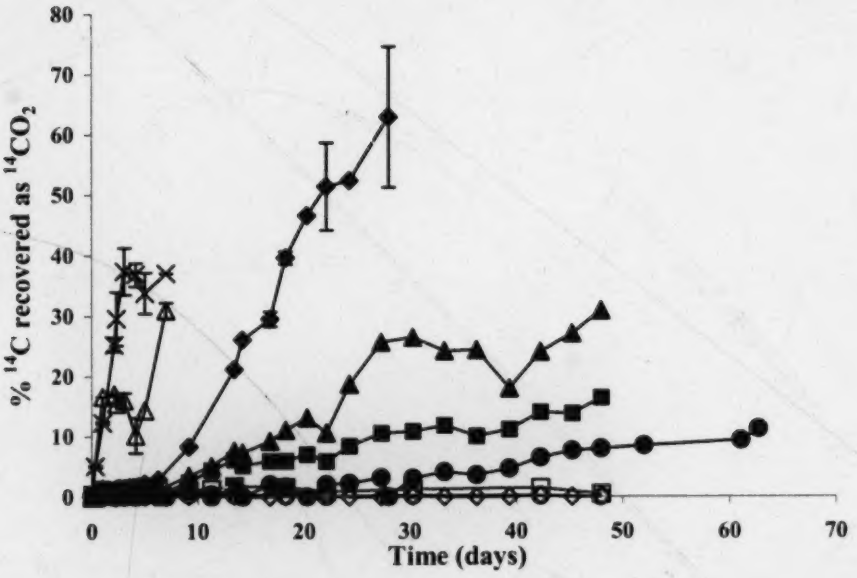

Figure 3.30. Mineralization by ANT 17 of various radiolabelled C substrates supplied at 1 mmol/L in Modified Mineral medium. Triplicate cultures were incubated at 1°, 8°, and 28°C with gyratory shaking, except as noted. Conditions varied as follows: [U-^{14}C]-glucose (×), *n*-[1-^{14}C]-hexadecane (□), [1-^{14}C]-palmitic acid (△), [3-^{14}C]-fluoranthene (○), [4,5,9,10-^{14}C]-pyrene (◇), and [9-^{14}C]-phenanthrene (▲), all incubated at 28°C with shaking; [9-^{14}C]-phenanthrene incubated at 28°C without shaking (■), [9-^{14}C]-phenanthrene incubated at 8°C with shaking (◆), and [9-^{14}C]-phenanthrene incubated at 1°C with shaking (●). Error bars (representing one standard deviation) are shown where appropriate, and otherwise omitted for clarity. Sterile control values did not exceed 1% $^{14}CO_2$ (not shown).

The phenanthrene results were contrary to expectation, as use of the modified Mineral medium did not result in decreased the lag time in the 28°C cultures with shaking (compared to Figure 3.29 under similar conditions). In addition the cultures did not achieve the same level of mineralization previously observed (50% radiolabel recovered as $^{14}CO_2$). As well, results from the static and shaking cultures were not as close in this experiment as they were in Figure 3.28. The 8°C phenanthrene cultures also had significantly shorter lag times than the results presented in Figure 3.28. In fact, mineralization occurred more rapidly and to a greater extent at 8° than at 28°C in this experiment.

Error bars are indicated for the 8°C phenanthrene culture series, and indicate good reproducibility between the triplicate cultures. Error bars were omitted from the 1° and both 28°C series, as the error was large once mineralization became significant. This was due to one of the cultures within each series beginning extensive mineralization ahead of the other cultures. One standard deviation of error was approximately equal to the mean values obtained for % ^{14}C recovered as $^{14}CO_2$ recovered at each time point. By the end of the time course, the mineralization values for the 1°C triplicates were 3, 4, and 27%; the static cultures at 28°C were at 9, 17, and 23%; and the shaking cultures were 9, 32, and 52%. These differences obviously exaggerated the level of error and confidence in these values. It is likely the triplicates for each condition would have attained the same level of mineralization if the incubations continued, however this was not done due to time constraints.

Despite the contradictory results compared to previous trials, mineralization did occur. At the final analysis time for each culture series, a radiolabel distribution was performed as described in Section 2.11.4 to determine the fate of the residual radiolabelled substrate. The results are presented in Table 3.6 and show the percentage of radiolabel found within each fraction, expressed as a mean (± standard deviations) of the triplicate cultures. The results of the radiolabel distribution for the sterile controls are also included. The percentages of radiolabel recovered as $^{14}CO_2$ are the values plotted in Figure 3.30. As most of these compounds were present at concentrations exceeding their solubility, the insoluble parent compounds (palmitic acid, phenanthrene, fluoranthene,

Table 3.6. Radiolabel distribution analysis conducted after incubation of ANT 17 with various radiolabelled substrates. Values represent the mean of triplicate culture analysis (± standard deviation) or single sterile controls.

	Percentage of initial radiolabel present in each fraction						
Radiolabelled substrate	**% CO_2**	**% Biomass**	**% Insoluble parent compound**	**% DCM extractable radiolabel**	**% Non-DCM extractable radiolabel**	**% Adsorbed radiolabel**	**TOTAL % radiolabel recovered**
[U-^{14}C]-glucose	37.1 (3.1)	9.8 (2.3)	- [a]	-	40.3 (1.2)	0.5 (0.0)	87.7 (3.9)
Sterile control	0.5	0.7	-	-	80.0	0.9	82.1
n-[1-^{14}C]-hexadecane	0.5 (0.2)	3.4 (2.0)	1.0 (0.3)	4.1 (1.1)	8.1 (4.4)	2.6 (0.6)	19.7 (3.6)
Sterile control	0.1	3.0	2.3	2.6	3.6	4.1	15.7
[1-^{14}C]-palmitic acid	30.9 (0.9)	1.2 (0.1)	39.0 (2.0)	-	31.9 (1.2)	2.0 (0.8)	105.0 (0.6)
Sterile control	0.0	2.1	39.6	-	43.6	2.2	87.5
[3-^{14}C]-fluoranthene	0.0 (0.0)	11.6 (0.4)	73.1 (7.3)	17.0 (4.5)	4.0 (0.8)	1.3 (0.4)	107.0 (4.6)
Sterile control	0.0	5.8	41.0	17.7	2.0	1.2	67.7
[4,5,9,10-^{14}C]-pyrene	0.0 (0.0)	53.5 (7.0)	41.8 (10.4)	15.4 (6.9)	0.8 (0.3)	1.5 (0.2)	113.0 (4.5)
Sterile control	0.0	38.5	51.0	18.5	3.9	1.4	113.3
[9-^{14}C]-phenanthrene at 28°C with shaking	30.8 (21.5)	7.5 (3.8)	14.0 (20.3)	6.4 (3.1)	8.6 (3.0)	1.0 (1.0)	68.3 (7.9)
Sterile control	0.0	24.0	64.9	4.0	2.7	3.4	99.0
[9-^{14}C]-phenanthrene at 28°C without shaking	16.3 (7.4)	11.3 (5.3)	63.8 (28.2)	3.2 (0.7)	7.1 (1.5)	0.9 (0.5)	102.6 (26.0)
Sterile control	0.0	3.0	70.3	11.4	3.9	1.4	90.0
[9-^{14}C]-phenanthrene at 8°C with shaking	62.8 (6.9)	6.4 (1.5)	22.8 (4.6)	4.0 (0.5)	13.2 (0.9)	0.9 (0.6)	110.1 (4.3)
Sterile control	0.4	11.6	26.8	8.7	6.4	3.9	57.8
[9-^{14}C]-phenanthrene at 1°C with shaking	11.2 (13.4)	7.7 (1.7)	26.9 (6.6)	1.4 (0.3)	10.5 (0.5)	2.1 (0.8)	59.8 (19.5)
Sterile control	0.6	6.0	53.5	5.0	2.5	3.4	71.0

[a] – not determined

and pyrene) were collected by filtration of the culture medium through glass wool. The percentage of radiolabel associated with biomass was assessed by harvesting the cells from filtered medium and lysing the pellet to account for both incorporated and cell-associated radiolabel. Small bacterium-sized particles of insoluble substrate may have passed through the filtration. Solvent extract of the acidified filtered medium was utilized to determine the percentage of radiolabel existing as non-polar DCM-extractable metabolites. Non-DCM-extractable polar metabolites were quantified in the aqueous phase. The percentage of radiolabel adhering to the glass surface of the culture flasks was also accounted for and was observed to be minimal in all cases.

The results of the glucose radiolabel distribution were as expected, as the cultures mineralized 37% to $^{14}CO_2$ (compared to the sterile control), with a modest but significant amount of radiolabel incorporated or associated with biomass. A corresponding decrease in the parent compound (glucose) within the culture medium was also observed when compared to the sterile control (in the non-DCM extractable column). Overall, the recovery of the radiolabelled glucose was satisfactory (in excess of 80%) and the totals for the inoculated and sterile control conditions were similar.

The results for the palmitic acid and hexadecane cultures again show the mineralization of palmitic acid but not hexadecane. Biomass was not a significant sink for radiolabel, in contrast with glucose. The total recovery of hexadecane was extremely poor, but consistent among all cultures and the sterile control. This suggests that loss of the substrate occurred during the sampling procedure, in which 1 mL of liquid and 2 mL of air were removed at each time point for $^{14}CO_2$ recovery. That is, radiolabel would have been removed with each liquid sub-sample and would neither have been replenished nor quantified in the radiolabel distribution. This loss was not observed with the insoluble substrates as large crystals were not removed due to the fine gauge needles used for sampling. Recovery of palmitic acid was satisfactory, however it should be noted that the amount recovered for the inoculated cultures was 20% greater than the sterile control. A significant amount of the radiolabel existed as insoluble substrate and remained unchanged comparing the sterile control and inoculated cultures. A reduction in DCM-insoluble radiolabel was observed and it is likely that this loss is due to the removal of soluble parent compound which was subsequently mineralized.

The radiolabel distribution data for the cultures containing pyrene and fluoranthene are similar. Good total recovery was achieved for all cultures except the fluoranthene sterile control. This may have been due to a methodological problem. No mineralization occurred as indicated by the lack of radiolabel recovered as $^{14}CO_2$. An increase in biomass-associated radiolabel was observed for both substrates, suggesting that ANT 17 was internalizing or sorbing the parent compound. The large value for biomass-associated label in the pyrene sterile control may have been due to radiolabel sorbed to or associated with inorganic precipitates in the medium which was not captured during the glass wool filtration and pelleted upon centrifugation. The increase in insoluble parent compound in the fluoranthene cultures (compared with the sterile control value) may have been due to biomass becoming trapped during the glass wool filtration. No significant differences were observed in the values for the DCM-extractable metabolites. The insoluble metabolites appeared to increase in the fluoranthene culture (but not significantly) while they decreased in the pyrene cultures.

The radiolabel distribution results from the cultures containing radiolabelled phenanthrene were comparable between cultures, with mineralization observed as described previously. The 28°C shaking cultures exhibited greater phenanthrene mineralization than the static cultures. An increase in biomass was observed for the static cultures but not for the shaking cultures (compared to the respective sterile controls). The large percentage of label recovered as "biomass" from the shaking sterile control might be due to increased sorption of radiolabel to inorganic precipitates within the medium caused by increased agitation associated with shaking. A dramatic reduction in parent compound within the shaking culture corresponds well with the large amount of mineralization. The total recoveries of radiolabel in the 28°C cultures were adequate except for the shaking inoculated cultures, as a lower total recovery was noted. The quantities of non-extractable metabolites increases slightly in the inoculated cultures under both shaking and static conditions. The DCM-extractable metabolite fraction was either unchanged or decreased with the shaking and static cultures respectively. The 1° and 8°C shaking cultures with radiolabelled phenanthrene had results similar to the 28°C cultures.

The radiolabel distribution work presented here was essentially a preliminary trial. Improvements should be devised to more accurately determine the fate of the radiolabel. For example, extended incubation times would allow for maximum mineralization where all triplicates would achieve the same level of mineralization, resulting in less variation in the radiolabel distribution determinations. As biomass apparently was trapped in the glass wool, an alternative method is required for determining radiolabel associated with biomass separate from residual insoluble parent compounds. Perhaps the initial concentration of compound could be reduced in conjunction with increased specific activity, to decrease the presence of insoluble substrate while maintaining a high amount of radioactivity for accurate determinations. Further analysis such as thin layer chromatography could be employed to determine the presence and number of metabolites in the DCM-extracted and non-extracted fractions. This would also allow the determination of the quantity of soluble parent compound remaining in the culture medium. Replicate sterile controls could also be utilized determine the quantities of radiolabel in each fraction with greater confidence. The low recovery of hexadecane might be accounted for by including an extra culture flask that is not sampled throughout the time course, to determine the loss of parent compound through sampling.

Overall, the 9-C atom of phenanthrene is mineralized and not incorporated or associated greatly with biomass. Importantly, phenanthrene mineralization occurs under cold conditions and in the absence of nutrient amendments, as these trials did not include N and P nutrient amendments (minimal nutrients amended from yeast extract in Modified Mineral medium). The presence of DCM extractable radiolabel is seen to decrease while the presence of non-extractable metabolites increases marginally. This would suggest the fate of the 9-C atom of phenanthrene degradation by ANT 17 is primarily to CO_2 and biomass accumulation. ^{14}C-labelled metabolites do not appear to accumulate. This was expected given the rapid and extensive growth of ANT 17 observed in earlier experiments when phenanthrene was supplied as the sole C source. Mineralization studies with phenanthrene radiolabelled in different positions would need to be conducted to confirm the absence of accumulating metabolites. ANT 17 does not mineralize fluoranthene or pyrene and the nature of the transformation observed is still unknown.

ANT 17 mineralization of palmitic acid but not hexadecane was expected as growth was observed as protein increase in liquid culture conditions with palmitic acid earlier.

4. CONLUSIONS AND FUTURE RESEACRH

4.1. Conclusions of this research

Overall, the research objectives of this thesis, outlined in Section 1.8, were achieved. The major conclusions of this thesis include:

a) ANT 17 is a psychrotolerant isolate identified as *Sphingomonas paucimobilis*;
b) the temperature optimum for growth is at 28°C;
c) the pH optimum for growth was 6.4 at 22°C, but the optimum pH at colder temperatures is less defined;
d) ANT 17 possesses a broad substrate range for degradation of a variety of petroleum and related compounds;
e) growth and degradation of crude oils and a refined product occurs under cold and nutrient limiting conditions;
f) ANT 17 was phenotypically stable and lacked plasmids; and
g) ANT 17 mineralizes phenanthrene to CO_2.

The remaining portion of this section will expand upon these conclusions and outline their significance to the field of cold climate bioremediation.

From the research conducted with *Sphingomonas* sp. isolate ANT 17, several conclusions can be made. Based on the taxonomic characterization studies, ANT 17 was identified as a *Sphingomonas paucimobilis* isolate. This Gram-negative bacterium is pigmented yellow due to the presence of the carotenoid nostoxanthin. Motility was variable in this isolate. This organism was also sensitive to various antibiotics. The unique FAME profile, compared to other organisms within this genus, suggests that ANT 17 has adapted in a specific manner to its cold environment in the Antarctic from where it was isolated, particularly increasing unsaturated fatty acids and reducing longer chain fatty acids. This organism is halotolerant, possibly UV resistant (most likely due to pigmentation), as well as copper and mercury tolerant.

ANT 17 grows optimally at 28°C, but grows at temperatures at least as low as 1°C, making this organism psychrotolerant. Growth at reduced temperatures occurred in both

nutrient medium and on hydrocarbons. The optimum pH for growth of ANT 17 is 6.4, which was a surprising characteristic as the pH of the isolation site was close to 9.0. However, the influence of pH appears to be prominent only at higher temperatures, whereas at lower temperatures ANT 17 grows equally well at more alkaline pH values.

Crude oil and refined product degradation studies indicated that ANT 17 possesses a broad substrate range for aromatic degradation. ANT 17 was capable of degrading crude oils under limiting conditions including low temperature, nutrient poor conditions, and reduced aeration and agitation. Amending the mineral medium with N and P nutrients did not significantly enhance biodegradation. This is an unexpected phenomenon as numerous cases have shown that the inclusion of nutrient amendments generally enhances biodegradation under cold and nutrient-limiting conditions (discussed in Section 1.5). The seemingly good biodegradation in unamended conditions increases the potential of this organism for *in situ* applications. The saturated fractions of the crude oils and the refined product tested were not degraded, although palmitic acid was mineralized. Generally, saturated contaminants are readily degraded by divers microbes and it is the degradation of the more recalcitrant aromatic compounds that is of concern. Therefore, it is likely that ANT 17 might react positively in a consortium of organisms, capable of degrading saturated contaminants, while it removes the aromatic compounds and possibly the partially oxidized *n*-alkanes.

Qualitative screens of 75 compounds indicated that ANT 17 possesses a broad substrate range. Saturated hydrocarbons were suitable as a growth substrate when provided individually in solid medium as the sole carbon source. Utilization of these compounds did not occur in liquid (tested individually) or when provided as the complete saturated fraction of crude oil. However, ANT 17 did grow on saturated compounds when supplied as vapours. Tolerance and even growth in the presence of these volatile compounds would be important for survival under conditions of decreased temperature, as the volatility of short-chain *n*-alkanes is reduced, thus increasing the toxicity of the compounds. ANT 17 was able to grow on, transform or solubilize a wide variety of aromatic compounds. This broad substrate range may be important for bioremediation of contaminated sites, as ANT 17 may initiate the biodegradation of various and otherwise recalcitrant compounds by oxidation or solublization, which could in turn increase the

bioavailability of the compounds for other organisms to complete the biodegradation process either to less harmful products or CO_2.

ANT 17 was phenotypically stable in its ability to degrade phenanthrene and transform dibenzothiophene. Plasmids could not be isolated from ANT 17 suggesting that the genes encoding for biodegradation enzymes are chromosomally encoded. Comparison of growth of ANT 17 on aromatic hydrocarbon degradation intermediates with known degradation pathways for phenanthrene biodegradation suggests that it may use a unique pathway with new intermediates or unique regulatory features that are different from the known phenanthrene pathways. ANT 17 was able to mineralize phenanthrene (labeled at the 9-C atom) to CO_2 under cold conditions. ANT 17 did not mineralize pyrene, fluoranthene or hexadecane. It appears that ANT 17 does not incorporate as much of these compounds serving as C sources compared to glucose (U-labeled), and it does not appear to excrete metabolites of aromatic degradation.

Overall, the ability of ANT 17 to degrade, transform or solubilize a wide variety of compounds (both aromatic and saturated) indicates a potential use for this organism for bioremediation of contaminated sites impacted by petroleum and related contamination. This organism can be described as being "hardy" under various limiting conditions, while maintaining the ability to carry out biodegradation. These conditions include cold temperatures, reduced nutrients, and reduced aeration. This organism may be a useful agent for bioremediation of contaminated sites in cold and limiting conditions. In addition, this organism could also be used as a model organism for comparison with other isolates from cold and limiting environments having the ability to biodegrade petroleum-related compounds.

4.2. Areas of future research

Prior to the use of ANT 17 for *in situ* applications of bioremediation of contaminated sites, more research is required, of both a practical and academic nature. The taxonomic characterization of ANT 17 could be expanded upon. Primarily the cell wall analysis could be expanded. Comparisons with other *Sphingomonas* spp. could be made in order to determine the nature of the membrane phospholipid adaptations ANT 17 makes compared to mesophilic isolates. Similarly, ANT 17 could be grown over a range of

temperatures to determine any changes in fatty acid composition in response to temperature reduction. The role of sphingoglycolipids in the outer membrane could be studied in order to determine their influence on cold tolerance and growth and in association with hydrophobic substrates.

UV resistance of ANT 17 could be clarified using better defined UV sources and compared to UV-sensitive isolates. Perhaps a mutant of ANT 17 could be generated lacking nostoxanthin production to determine the influence of this carotenoid on UV resistance. Other metal resistances could be assessed, especially lead tolerance, a common co-contaminant at historical gasoline spill sites. The tolerance of ANT 17 to cycles of freezing and thawing could be determined under conditions more closely resembling *in situ* conditions through the use of microcosms. Other studies could include growth at temperatures below 0°C. Upon the discovery of a suitable buffering system for medium at pH values above 8, further expansion of the pH range for ANT 17 should be assessed, particularly under cold conditions. The role of inoculum density on ANT 17 growth could also be expanded, possibly beginning with the determination of the presence of homoserine lactone cell density signals, as found in other Gram-negative bacteria.

Studies of a more practical nature for biodegradation should include the combination of various limiting conditions to more closely parallel those found *in situ*, as generally a cold site may also be nutrient limiting and affected by other potentially inhibitory conditions for biodegradation. These studies could be combined with microcosm work, possibly using consortia from other contaminated sites to determine the role of ANT 17 in biodegradation under natural conditions.

Finally, ANT 17 studies could continue in the investigation of phenanthrene degradation, elucidating the pathway and the metabolites produced prior to mineralization, the enzymes involved, the genes required and their regulation. Similarly, other compounds and pathways used by ANT 17 could be analyzed. For instance, ANT 17 could be directly compared to *S. paucimobilis* EPA505 (Mueller *et al.*, 1990) as similar coloured transformation products of fluoranthene degradation were observed. Although ANT 17 did not mineralize fluoranthene, these two isolates could be compared in order to determine if the intermediates produced are the same.

Overall, this organism possesses some unique and interesting features with respect to cold climate biodegradation. *S. paucimobilis* ANT 17 has demonstrated the potential for biodegradation of a wide variety of petroleum-related compounds under limiting conditions. It is hoped that further research with this isolate is conducted in order to determine the full range and capabilities this bacterium may have for bioremediation. Use of this off continent bacterium in field studies will require regulatory agency permission. The similarity of ANT 17 to other known *Sphingomonas* spp. should enable that decision. With further studies, this organism may prove to be useful for *in situ* applications of bioremediation to contaminated sites.

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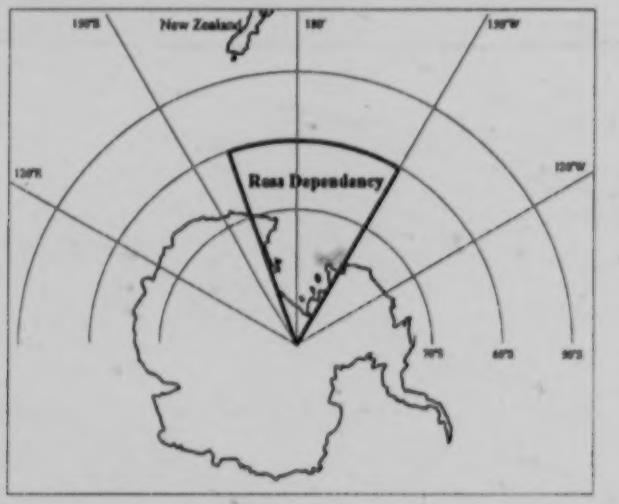
APPENDICES

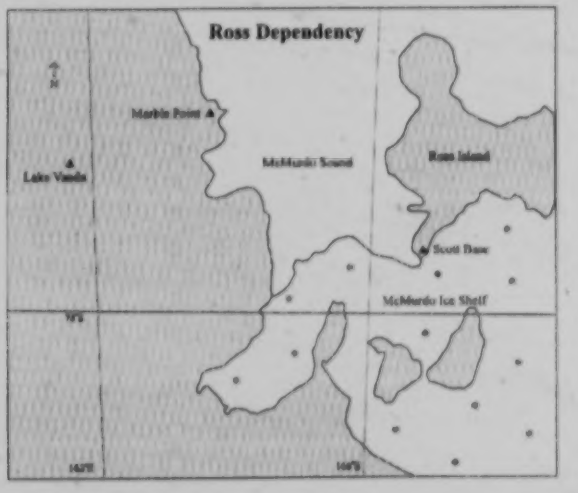
APPENDIX A. ISOLATION OF ANT 17

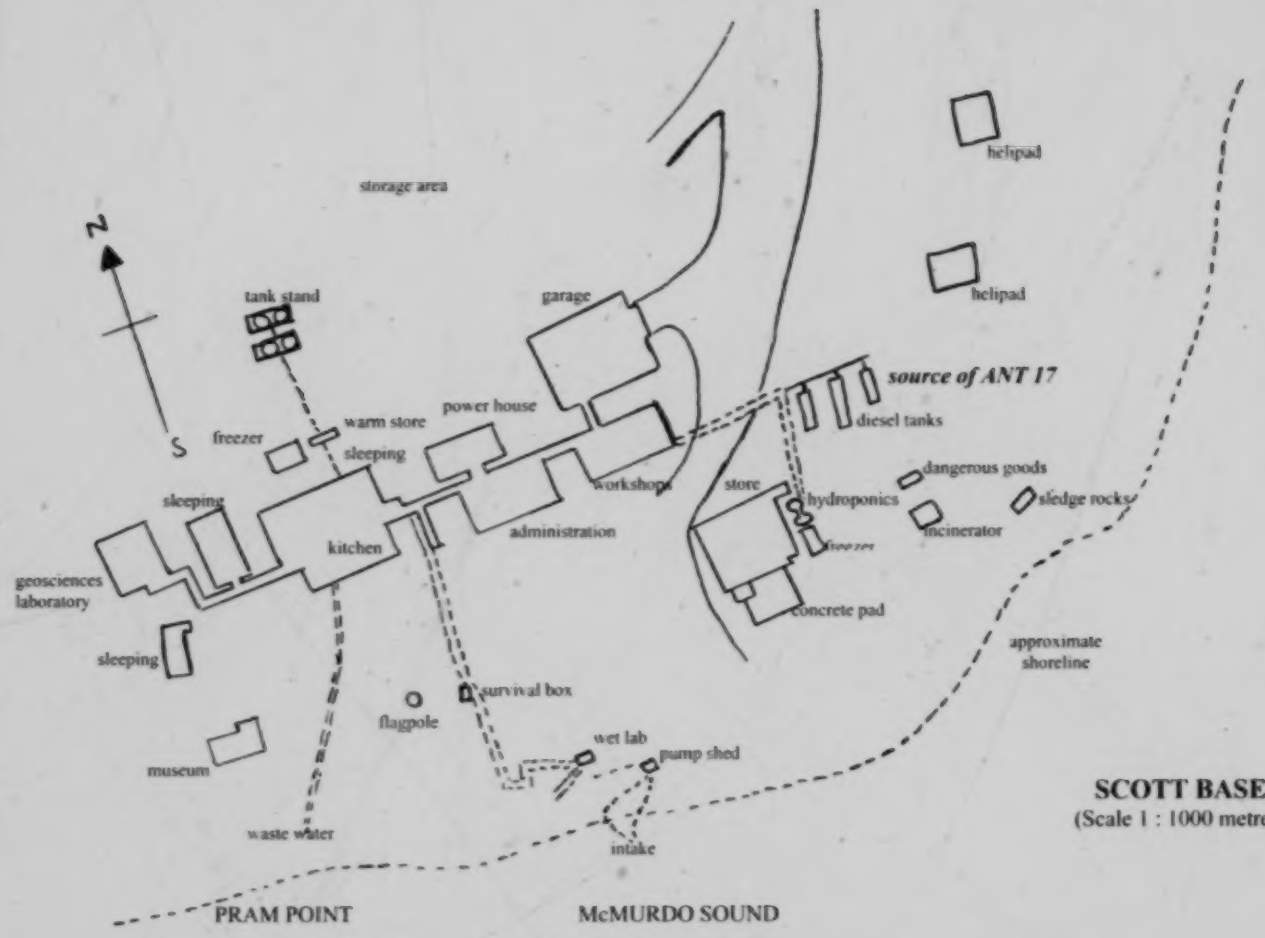
A.1. Map of Antarctica and the Ross Dependency

This figure shows the geographical region of the Ross Dependency within Antarctica (provided by J. Aislabie, 1997, personal communication).



This figure illustrates the location of Scott Base, Marble Point and Lake Vanda within the Ross Dependency (provided by J. Aislabie, 1997, personal communication). Marble Point and Lake Vanda were other areas of study and will not be discussed here.





SCOTT BASE
(Scale 1 : 1000 metres)

A.2. Plan view of Scott Base

A.3. Soil data from isolation site

A.3.1. Hydrocarbons identified from contaminated soil

Concentrations of PAHs in ANT 17 isolation soil

Compound	*Approximate concentration (ng/g)*
naphthalene	200
acenaphthylene	200
acenaphthene	350
fluorene	1000
phenanthrene	430
anthracene	20
fluoranthene	5
pyrene	5
benz[a]anthracene	3
chrysene	4
benzo[b]fluoranthene	3
benzo[k]fluoranthene	1
benzo[a]pyrene	1
indeno[1,2,3-cd]pyrene	1
dibenz[ah]anthracene	0.2
benzo[ghi]perylene	1
Total unsubstituted PAH	**2224**

(this data was provided by J. Aislabie, 1997, personal communication)

Hydrocarbon contaminants identified in contaminated soil from Scott Base, Antarctica (adapted from Aislabie *et al.*, 1998)

Hydrocarbons identified	**Amount present (μg per g dry weight soil)**	
	Sample source	
	Below generator	Kitchen fuel spill
Alkanes		
n-C_9	<2	486
n-C_{10}	<2	1740
n-C_{11}	263	2170
n-C_{12}	554	1780
n-C_{13}	653	1016
n-C_{14}	443	493
Aromatics		
C_2-benzenes	<2	<2
C_3-benzenes	<2	360
C_4-benzenes	<2	836
Naphthalene	<2	<2
C_1-naphthalene	<2	<2

A.3.2. Total heterotrophs and hydrocarbon degraders enumerated in samples from Scott Base

Data were obtained from Balks *et al.* (1998). Plate counts for heterotrophs and MPN analysis for hydrocarbon degraders incubated at 16°C. JP8 was supplied as the sole C and energy source for the enumeration of hydrocarbon degraders (Aislabie *et al.*, 1998). The data for the isolation site of ANT 17 have not been provided (i.e. under the diesel tanks), however, these sites are representative of JP8-contaminated sites at Scott Base. The kitchen site is contaminated as a result of a JP8 spill from a small tank. The control site was away from vehicle tracks.

Sample	Location	TPH[a] µg/g dry weight soil	Number of culturable heterotrophs per g dry weight soil	MPN of hydrocarbon degraders per g dry weight soil
Site 1	***Drum storage site***			
1	Surface (0-2 cm)	9070	3.63×10^6	1.2×10^7
2	2-10 cm (wetlayer)	4960	6.21×10^6	1.69×10^8
3	10-20 cm	2062	6.1×10^6	1.7×10^7
4	20-30 cm	1922	1.32×10^6	2.52×10^6
Site 2	***Kitchen site***			
6	Surface (0-2 cm)	25	4.6×10^7	2.5×10^6
7	2-5 cm	390	7.5×10^6	3.5×10^6
8	5-20 cm	8	6.9×10^6	3.0×10^4
Site 3	***Control site on hill***			
10	Surface (0-2 cm)	<2*	4.7×10^5	1.3×10^4
11	1-7 cm	<2	8.74×10^6	1.1×10^4
12	15-20 cm	<2	4.4×10^4	3.6×10^3
13	35-38 cm	<2	1.96×10^3	2.44×10^3

[a] Total Petroleum Heterotrophs

A.3.3. Soil parameters

The soil sample locations are the same as those described in Appendix A.3.2. These data were obtained from Balks *et al.* (1998).

Sample	Water % dry wt	pH (water)	Total C (%)	Total N (%)	Total P (%)	Total S (%)	Electrical conductivity (mS/cm)	Mineral N (mg/kg)	Olsen-P (mg/kg)
1	2.0	8.1	3.63	0.01	0.19	0.10	0.59	4.6	7
2	5.6	9.3	2.56	0.01	0.17	0.06	0.17	5.4	9
3	3.6	9.6	0.90	0.00	0.17	0.02	0.07	6.0	3
4	4.6	9.6	0.85	0.00	0.22	0.03	0.13	4.1	3
6	13.1	8.9	0.97	0.05	0.23	0.04	0.31	123.3	114
7	10.8	8.9	0.58	0.03	0.19	0.03	0.28	16.2	28
8	13.9	9.4	0.45	0.02	0.20	0.03	0.23	9.0	19
10	2.4	8.9	0.07	0.01	0.20	0.06	1.24	20.2	12
11	3.5	9.4	0.08	0.01	0.21	0.02	0.33	7.5	13
12	7.5	9.6	0.04	0.01	0.19	0.01	0.09	6.7	11
13	9.8	9.5	0.07	0.01	0.17	0.02	0.13	6.6	13

APPENDIX B. MEDIA AND SOLUTIONS

All media were prepared using doubly distilled water (ddH_2O), and sterilized by autoclaving at 121°C for a minimum of 15 min (depending on volume). Liquid cultures were allowed to cool completely prior to inoculation. For plates, the medium was allowed to cool in a water bath to approximately 50°C. Once cool, volumes of approximately 25 mL were poured into petri plates and allowed to set and dry overnight at room temperature prior to use. All media were stored at 4°C in the dark until use. All components are listed per Litre of ddH_2O, except where specifically noted.

B.1. Media for general culture conditions

Nutrient agar plus 0.5% glucose (NAG) (Mueller *et al.*, 1990)

Bacto-peptone (Difco)	5 g
Bacteriological beef extract (Difco)	3 g
Bacto-agar (Difco)	15 g
pH 6.8	

This mixture was prepared in 900 mL ddH_2O and sterilized by autoclaving. In order to achieve a final concentration of 0.5% glucose, a 10X stock was prepared and autoclaved separately:

glucose	5 g in 100 mL ddH_2O

When both solutions were cooled to 50°C after sterilization, the glucose solution was added to the remaining medium components, mixed thoroughly by swirling and poured into plates.

Nutrient broth plus 0.5% glucose (NBG)

Prepared as for NAG, with the omission of agar.

Luria Bertani (LB) agar

Bacto-tryptone (Difco)	10 g
Yeast extract (Difco)	5 g
NaCl	10 g
Bacto-agar (Difco)	15 g
pH adjusted to 7.5	

B.2. Differential media and media for taxonomic determinations

Blood agar

Bacto-tryptose (Difco)	10 g
Bacteriological beef extract (Difco)	3 g
NaCl	5 g
Bacto-agar (Difco)	15 g

This mixture was sterilized by autoclaving and cooled to 50°C. Sterile defibrinated sheep blood (at room temperature) was aseptically added to achieve a final concentration of 5% v/v.

Antibiotic Resistance tests

Kanamycin

Kanamycin was added as a filter sterilized stock solution of 25 mg/mL, in ddH_2O to cooled sterile LB medium to achieve a final concentration of 50 μg/mL.

Rifampicin

Rifampicin was added as a filter sterilized stock solution of 25 mg/mL in methanol to cooled sterile LB medium to achieve a final concentration of 150 μg/mL.

Antibiotic disk diffusion assays

Commercially prepared disks (Difco) containing antibiotics were placed in the centre of inoculated PCA plates to qualitatively assess growth in the presence of the antibiotic. The following antibiotics were used:

chloramphenicol	30 μg
novobiocin	30 μg
streptomycin	10 μg
tetracycline	30 μg
penicillin G	10 units

Salt tolerance medium

TSB was amended with various salt concentrations (0% to 8% w/v NaCl) and dispensed as 10 mL per tube.

Bacto motility agar
(Difco Manual, 1984)

Bacto-tryptone (Difco)	10 g
NaCl	5 g
Bacto agar (Difco)	5 g

Dispensed into tubes, sterilized and cooled quickly in an upright position in a water bath at room temperature.

Metal resistance (Nieto *et al.*, 1989)

Mineral medium (Appendix B.3.) supplemented with glucose and agar was used to avoid the problem of sequestering metal ions in a richer medium.

Mineral medium	1 L
glucose	2 g
Bacto-agar (Difco)	15 g

The medium was dispensed into tubes, sterilized by autoclaving, and held at 50°C. Then the stock metal solution was added, mixed briefly by vortex and poured into Petri plates.

Metal stock solutions were dissolved in ddH_2O, filter sterilized and stored at 4°C until needed. $HgCl_2$ (273 mM) and $CuSO_4 \cdot 5H_2O$ (600 mM) were utilized to give final metal concentrations ranging from 0 mM to 40 mM.

B.3. Media used to assess growth on petroleum-related compounds

Mineral medium (Foght and Westlake, 1988)

K_2HPO_4	4.4 g
KH_2PO_4	1.7 g
NH_4Cl	2.1 g
100X salt solution	10 mL

15 g Noble agar (Difco) added for solid medium

100X salt solution

$MgSO_4 \cdot 7H_2O$	19.5 g
$MnSO_4 \cdot H_2O$	5.0 g
$FeSO_4 \cdot 7H_2O$	5.0 g
$CaCl_2 \cdot 2H_2O$	0.3 g
ascorbic acid	1.0 g

filter sterilized and stored at 4°C

Westlake N,P 50X stock (Foght *et al.*, 1999)

K_2HPO_4	25 g
NH_4Cl	50 g
KNO_3	100 g

heat sterilized and stored at 4°C

Bushnell – Haas (Difco Manual, 1984)

$MgSO_4 \cdot 7H_2O$	0.2 g
$CaCl_2 \cdot 2H_2O$	0.02 g
KH_2PO_4	1 g
K_2HPO_4	1 g
NH_4NO_3	1 g
$FeCl_3 \cdot 6H_2O$	0.05 g
pH	7.0

Mueller Mineral salts (Mueller *et al.*, 1989)

$(NH_4)_2SO_4$	1 g
K_2HPO_4	0.8 g
KH_2PO_4	0.2 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$CaCl_2 \cdot H_2O$	0.1 g
$FeCl_3 \cdot 6H_2O$	0.005 g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.001 g

pH adjusted to 7.0 with 0.1M HCl and sterilized prior to adding C source

Tween® 80 (J. T. Baker Chemical Co., Phillipsburg, NJ)

Add 185 µl per litre of medium to achieve final concentration to 200 mg/L.

BYP (Foght and Westlake, 1988)

Yeast extract (Difco)	1 g
Proteose peptone #3 (Difco)	1 g
B+N,P	1 L
Bacto-agar (Difco)	15 g

B+N,P (Foght and Westlake, 1988)

K_2HPO_4	0.5 g
NH_4Cl	1.0 g
Na_2SO_4	2.0 g
KNO_3	2.0 g
$MgSO_4 \cdot 7H_2O$	0.2 g
$FeSO_4 \cdot 7H_2O$	trace

Vapour plates (for volatile solid or liquid C sources)

Vapour phase C sources were supplied by placing a few crystals of a solid compound (e.g. naphthalene) onto the lid of a mineral agar petri plate after inoculation (care must be taken to keep the lid of the plate down at all times to prevent the crystals falling onto the agar surface). For a liquid C source, a few drops of the compound was placed into a sterile Durham vial stuffed with glass wool and secured to the lid of the plate with tape surface-sterilized with ethanol. All vapour plates were sealed with parafilm and placed in separate bags to prevent vapour contamination. Control plates lacking the compound were also included.

Overlay plates

Approximately 0.1 g of the compound to be tested was dissolved in 3 to 6 mL DMF and filter-sterilized through a syringe tip filter (Millex FG, 0.22 μm) into a sterile glass vial with a Teflon lined cap. For the overlay medium, Bushnell-Haas medium with 1% agarose and 0.01% yeast extract was dispensed into tubes (4 mL per tube) prior to autoclaving. After autoclaving, the tubes were held at 60°C. One hundred to two hundred microlitres of the sterile compound solution were added to the tube, quickly mixed and poured onto prepared mineral medium plates. The overlay was quickly swirled on the plate surface thus creating an even overlay of the modified Bushnell-Haas with the test compound. The compound precipitates once added to the molten agarose suspension, which creates an opaque overlay. This allows growth and zones of clearing around colonies to be observed on the plate surface. Prepared plates were allowed to dry overnight prior to use.

Spray plates

To test the ability of a compound to serve as a C source, a mineral plate lacking a C source was inoculated then sprayed with an ethereal solution of the desired compound using an aerosolizer (JetPak, Sherwin-Williams Co., OH). Approximately 0.1 g of the desired compound was placed into a 13 x 100 mm glass test tube and dissolved in ether. The suspension was sprayed evenly over the plate surface to serve as a C source.

To test for transformation of a compound, the organism was first grown on a BYP plate until visible colonies were observed. The plate was then sprayed and observed for up to 24 h to determine if a coloured transformation product was produced. All plates were incubated in the dark to prevent photooxidation.

Compounds suspended within agar

Compounds in a concentrated DMF stock solution were added to molten mineral agar (15 mL/tube, sterilized by autoclaving and held at 60°C in a waterbath) to achieve a final concentration of 2.5 mmol/L. After mixing the tubes by vortex, plates were poured and allowed to set prior to inoculation.

B.4. Buffers and solutions

10 mM phosphate buffer

100X phosphate buffer stock

KH_2PO_4 (1M)	20.3 g in 150 mL ddH_2O
K_2HPO_4 (1M)	43.5 g in 250 mL ddH_2O

The monopotassium phosphate solution is added to the dipotassium phosphate solution until a pH of 7.2 to 7.3 is obtained. This stock is then diluted 1:100 with ddH_2O to achieve a final concentration of 10 mM phosphate.

Buffered media for pH analysis

(Gerhardt *et al.*, 1994)
This mixed buffer system involved varying the amount of 0.2 M Na_2HPO_4 and 0.2 M NaH_2PO_4. These two solutions were dispensed in appropriate volumes as indicated below and brought to the desired final volume of 50 mL with 12.5 mL TSB solution and 12.5 mL ddH_2O to yield buffered QTSB.

pH	0.2 M NaH_2PO_4 mL	0.2 M Na_2HPO_4 mL
5.8	23.0	2.0
6.0	21.9	3.1
6.2	20.4	4.6
6.4	18.4	6.6
6.6	15.6	9.4
6.8	12.8	12.2
7.0	9.8	15.2
7.2	7.0	18.0
7.4	4.8	20.2
7.6	3.3	21.7
7.8	2.1	22.9
8.0	1.3	23.7

In order to achieve pH conditions buffered above 8.0, a Tris-HCl buffering system was required. Twenty-five millitres of 0.2 M Tris and 12.5 mL TSB were combined with 0.2 M HCl and ddH_2O in the quantities listed below.

pH	0.2 M HCl mL	ddH_2O mL
8.2	11.0	1.5
8.4	8.3	4.2
8.6	6.1	6.4
8.8	4.1	8.4
9.0	2.5	10.0

APPENDIX C. REAGENTS FOR TAXONOMIC DETERMINATIONS AND CELL STAINING

Acid Fast stain (Kinyoun's method; Norrell and Messley, 1997)

Kinyoun's carbol fuchsin

basic fuchsin	4 g
95% ethanol	20 mL
ddH_2O	100 mL (added slowly while shaking)
phenol	8 mL

Acid alcohol

concentrated HCl	3 mL
95% ethanol	97 mL

Methylene Blue

methylene blue	0.3 g
ddH_2O	100 mL

A loopful of grown broth culture is smeared onto a glass slide and heat fixed. The slide is flooded with Kinyoun's carbol fuchsin for 5 min and rinsed with ddH_2O. The acid alcohol is used to rinse the slide just until no further stain is removed and is followed immediately by a rinse with ddH_2O. Methylene blue is used to counterstain for 1 min and the preparation is then observed under oil immersion microscopy.

Metachromatic granule stain (Loeffler's procedure, Norrell and Messley, 1997)

Loeffler's methylene blue

methylene blue	0.3 g
95% ethanol	30 mL
ddH_2O	100 mL

A heat fixed smear is flooded with Loeffler's methylene blue for 3 min, rinsed with water and air dried. The preparation is then observed under oil immersion microscopy. Dark blue granules can be observed against a light blue cytoplasm.

Capsule stain (Anthony's method, Norrell and Messley, 1997)

1% aqueous crystal violet

crystal violet	1 g
ddH_2O	100 mL

20% cupric sulfate

cupric sulfate	20 g
ddH_2O	100 mL

An air dried smear of cells on a glass slide is stained for 2 min with the 1% aqueous crystal violet solution. The 20% cupric sulfate solution is then used to gently rinse the slide. After draining and blotting the slide to dryness, the preparation is observed under oil immersion microscopy. Cells stain a dark blue or purple while capsules are a light blue.

APPENDIX D. REAGENTS FOR MOLECULAR STUDIES

Plasmid DNA preparation procedure and reagents (below)
The procedure was as described by Kieser (1984) with slight modifications as indicated. Small scale preparations are performed by harvesting 1 mL of cells from a TSB grown culture. The cell pellet is resuspended in 0.5 mL lysis buffer. Lysozyme is omitted, as it is unnecessary for Gram-negative bacteria. Next, 0.25 mL lysis solution is added, mixed by gentle inversion, incubated at 55°C for 20 min and cooled to room temperature. Next, 0.5 mL potassium acetate solution is added and the lysate incubated on ice for 10 min, followed by microfugation to clarify the lysate. The supernatant is transferred to a new tube. DNase-free RNase A (Sambrook *et al.*, 1989) is added and allowed to incubate for 2 h at 37°C. The procedure then continues through two phenol-chloroform extractions, mixing and removing the aqueous phase both times. The addition of sodium acetate is omitted. One volume of isopropanol is used to precipitate the DNA instead of two volumes of 95% ethanol (at the first precipitation step). This is incubated overnight at –20°C or for 30 min at –70°C. The plasmid DNA is collected by centrifugation, washed with cold 70% ethanol, incubated at –20°C for 1 h and reharvested. The plasmid DNA pellet is then air dried and resuspended in TE and stored at either 4° or -20°C until analyzed.

Lysis buffer

sucrose	0.3 M
Tris·HCl (pH 8)	25 mM
Na_2EDTA	25 mM

pH 8
heat sterilized

Lysis solution

NaOH	0.3 N
SDS	2% w/v

solution made fresh

Potassium acetate solution

potassium acetate (5 M)	60 mL
glacial acetic acid	11.5 mL
ddH_2O	28.5 mL

pH 4.8
filter sterilized and stored at 4°C

DNase-free RNase (pancreatic RNase, Sigma)
(Sambrook et al., 1989)
The enzyme is dissolved in 0.01 M sodium acetate (pH 5.2) at 10 mg/mL. The solution is heated at 100°C for 15 min and cooled slowly to room temperature. The pH is adjusted by adding 0.1 volumes of 1 M Tris·HCl. The enzyme solution is dispensed into approximately 1-mL aliquots in sterile microfuge tubes and stored at –20°C until used. The stock is added to the cell lysate at a final concentration 0.1 mg/mL .

phenol-chloroform

crystalline phenol (Anachemia, Montreal, PQ, >99% purity)	5 g
chloroform	5 mL
8-hydroxyquinoline	5 mg
ddH_2O	1 mL

TE buffer (pH 8)

Tris·HCl (pH 8)	10 mM
Na_2EDTA	1 mM

Chromosomal DNA preparation procedure and reagents (below)
Cells are grown to logarithmic phase and harvested by centrifugation. The cell pellet is resuspended in saline-EDTA solution and harvested again. Upon resuspension of the washed pellet in saline-EDTA, the cells are transferred to a 250-mL flask. The cells are lysed in 3 mL of 25% (w/v) SDS solution, incubated for 10 min at 60°C in a waterbath and allowed to cool to room temperature. Seven millilitres of fresh sodium perchlorate is added and mixed well. An equal volume of chloroform-isoamyl alcohol mixture is added and the suspension is swirled gently on a platform shaker for 30 min at 100 rpm. Lumps can be removed from the suspension by diluting with 5 to 10 mL SSC and continued shaking for 15 to 30 min. The aqueous and organic phases are separated by gentle centrifugation, and the upper aqueous phase is removed, avoiding interfacial material, and retained (volume is noted). The DNA is precipitated by adding 2 volumes of 95% ethanol, mixed gently by inversion and harvested by centrifugation. Excess ethanol is drained from the pellet, and the pellet is dissolved in 9 mL 2X SSC. DNase-free RNase is added to a final concentration of 0.2 mg/mL, and incubated at 37°C for 30 min with occasional mixing. Stock EDTA is added to a final concentration of 10 mM, pronase to a final concentration of 0.2 mg/mL, mixed and incubated for 60 min at 37°C with occasional mixing. The final volume is noted, 2 volumes of 70% ethanol are added, mixed by inversion, incubated overnight at –20°C and the resulting DNA precipitate is collected by centrifugation. Excess ethanol is drained and the DNA is dissolved in a minimal volume of TE buffer (pH 8) or 2X SSC.

saline-EDTA

NaCl	0.15 M
Na_2EDTA	0.10 M

pH 8, store at 4°C

25% SDS (w/v)
in ddH_2O
store at room temperature

Sodium perchlorate

sodium perchlorate	31.6 g
ddH_2O	45 mL

made fresh daily

chloroform-isoamyl alcohol
mixed 24:1 (v/v)
made fresh daily

SSC 20X stock

NaCl	0.15 M
sodium citrate	0.015 M

pH 7.2

DNase-free RNase (10 mg/mL stock solution in 0.01 M sodium acetate)
prepared as described for plasmid DNA preparation
added to a final concentration of 0.2 mg/mL

stock EDTA solution

Na_2EDTA	0.05 M

pH 8
store at 4°C

Pronase (nuclease-free; Calbiochem, San Diego, CA)
20 mg/mL stock in sterile ddH_2O
store at –20°C
added to a final concentration 0.2 mg/mL

Gel electrophoresis

50X TEA stock solution

Tris·HCl (pH 8)	242 g
Na_2EDTA	18.61 g
Glacial acetic acid	57 mL
ddH_2O	to 1 L

(pH 8)

0.7% agarose

agarose (UltraPure, Life Technologies Inc., MD)	0.35 g
1X TEA	50 mL

heat in microwave until molten, cool and pour into gel apparatus

5X loading dye (Sambrook *et al.*, 1989)
50% glycerol (in ddH_2O)
0.1 M Na_2EDTA, pH 8
0.25% bromophenol blue
0.25% orange G (optional RNA marker)
0.25% xylene cyanol (optional high molecular weight DNA marker)
filter sterilize solution, dispense into approximately 1-mL aliquots in sterile microfuge tubes and store at –20°C

CHEF-PFGE

General protocol (see below for reagent list)

Agarose plug sample preparation

Overnight cultures are pelleted by centrifugation and the pellets are resuspended in an equal volume of 0.25 M EDTA (pH 8). Lysozyme is added to a final concentration of 0.5 mg/mL. The suspension is incubated at 37°C for 30 min. During this time a 1% solution of low gelling agarose in 0.25 M EDTA (pH 8) is melted and allowed to cool to 50°C. This is then added to the cell-enzyme suspension to achieve a final agarose concentration of 0.75%, adding additional 0.25 M EDTA if required. The suspension is pipetted into the CHEF chamber comb and allowed to set at 4°C for 20 min.

The agar plugs containing the cell-enzyme suspension are placed into a Petri dish with enough LET buffer to cover the plugs. This is incubated overnight at 37°C. The LET buffer is removed and the plugs are washed three time for 15 min each in 0.05 M EDTA (pH 8). SDS solution is then added to the plugs and this is then allowed to incubate overnight at 50°C. The SDS solution is removed and the plugs are washed for 15 min and again overnight in 0.05 M EDTA (pH 8) at room temperature. A final wash in 0.05 M EDTA (pH 8) is conducted for 15 min at room temperature and the samples can then be stored at 4°C in this buffer until used (up to 1 year).

PFGE (Netolitsky, 1995)

The agarose plugs containing the lysed cell samples are cut with a glass coverslip and placed into the wells of a prepared gel up to 90% of the well height. Similarly the desired amount of λ ladder DNA is also cut and placed into the well(s). The wells are then filled with a molten solution of 1% agarose in 0.5X TBE and allowed to set. The gel is placed in the apparatus and rested on the gel stops. The gel is covered with 1 to 2 mm of 0.5 TBE (running buffer). Liquid samples can be added to the gel at this time. A pump is used to circulate and cool the buffer (3 L required) through a cooling chamber and the gel apparatus at a flow rate of 1L/min. This will allow the gel to be run at a temperature of 14°C. The gel should be run for 10 min prior to the pump being turned on to facilitate entry of the samples into the gel. For this analysis the gel apparatus was run for 24 h with a ramped pulse of 165 V increasing over time from 5 to 80 s. The gel can then be stained and visualized for as other gels.

0.5 M EDTA (pH 8)

$Na_2EDTA\,2H_2O$	181.6 g
ddH_2O	800 mL

pH adjusted to 8.0 with ~20 g NaOH pellets and sterilized by autoclaving

0.25 M EDTA (pH 8)
dilute 0.5 M EDTA solution 1:2 and confirm pH

0.05 M EDTA (pH 8)
dilute 0.5 M EDTA solution 1:10 and confirm pH

Lysozyme (Boehringer Mannheim)
stock 50 mg/mL (in ddH_2O)
store at –20°C

Low gelling agarose
Sigma A4018 type VII
$\leq$ 0.01% sulfate
$\geq$ 200 g/cm^2 strength at 1%
gel point = 26° to 30°C at 1.5%
melting temperature $\leq$ 65°C
EEO $\leq$ 0.1

LET buffer

EDTA (pH 8)	0.45 M
Tris (pH 7.5)	0.01 M
2-mercaptoethanol	7.5 % (can be omitted, K.L. Roy, personal communication, 1997)

SDS solution

Tris (pH 7.5)	0.01 M
EDTA (pH 8)	0.45 M
SDS	1% w/v
proteinase K	1 mg/mL

Proteinase K (Boehringer Mannheim)
20 mg/mL (in ddH_2O)
store at –20°C

TBE (5X stock)

Tris	54 g
Boric acid	27.5 g
0.5 M EDTA (pH 8)	20 mL
ddH_2O	980 mL

(store at room temperature)

λ DNA ladder (New England Biolabs, Beverly, MA)
molecular weight marker, 50 μg/mL
50 to 1000 kb
supplied in an agarose plug within a syringe dispenser (10 mM Tris-HCl, pH 8, 1 mM EDTA, 50% glycerol), ready to cut and insert into gel wells

Agarose gel
1% in 0.5X TBE

low gelling agarose	2.5 g
TBE (0.5X)	250 mL

melt and pour into apparatus, let set

APPENDIX E. CHEMICALS USED

E.1. Chemicals, suppliers and purities

Chemical	Supplier, Location	Purity (if indicated)
Petroleum and related compounds		
JP8	Esso Avitat, Edmonton, AB	
PBCO	Atlantic Richfield Co., Seattle, WA	
NW	Imperial Oil, Calgary, AB	
ASMB	Environment Canada, Ottawa, ON	
Diesel #2	Texaco, Houston, TX	
anthracene	Aldrich, Milwaukee, WI	>99.9 %
9-anthraldehyde	Sigma, St. Louis, MO	
anthrone	MC+B, East Rutherford, NJ	
benzene	Fisher, Fair Lawn, NJ	pesticide grade
benzoic acid	MC+B	
benzophenone	Fisher	
biphenyl	Eastman, Rochester, NY	
carbazole	Lancaster, Windham, NH	98 %
catechol	Aldrich	>99 %
camphor (D+)	Aldrich	
9-chloroanthracene	Aldrich	75 %
1-chloronaphthalene	Eastman	
chrysene	Aldrich	98 %
dibenzofuran	Aldrich	
dibenzothiophene	Aldrich	98 %
2,2'-dihydroxybiphenyl	Sigma	
3,4-dihydroxybenzoic acid	Aldrich	97 %
1,2-dihydroxynaphthalene	Aldrich	
2,3-dihydroxynaphthalene	Aldrich	98 %
1,2-dimethylnaphthalene	Terochem, Edmonton, AB	
1,3-dimethylnaphthalene	K + K, Plainview, NY	
1,4-dimethylnaphthalene	K + K	
1,5-dimethylnaphthalene	K + K	
1,7-dimethylnaphthalene	Terochem	
1,8-dimethylnaphthalene	Terochem	
2,3-dimethylnaphthalene	K + K	
2,6-dimethylnaphthalene	K + K	
n-dodecane	Aldrich	>99 %
2-ethylnaphthalene	Sigma	
fluoranthene	Aldrich	98 %
fluorene	Aldrich	98 %
9-fluorenone	Aldrich	98 %
gentisic acid	Sigma	
n-hexadecane	Fisher	99.8 %

n-heptadecane	ICN, Plainview, NY	99 %
hexadecanoic acid	Aldrich	99 %
n-hexane	various	
hexanoic acid	Fisher	
4-hydroxybenzoic acid	Eastman	
1-hydroxy-2-naphthoic acid	Aldrich	99 %
2-hydroxy-3-naphthoic acid	TCI, Tokyo	
indole	MC+B	
isoquinoline	Sigma	
2-methylanthracene	Aldrich	
2-methylanthraquinone	Aldrich	95 %
4-methylbenzoic acid	Pfaltz + Bauer, Stamford, CN	
1-methylnaphthalene	Aldrich	95 %
2-methylnaphthalene	Aldrich	
2-methyl-1,4-naphthoquinone	Aldrich	98 %
4-methylsalicylic acid	Aldrich	99 %
1-naphthylacetic acid	Aldrich	97 %
naphthalene	Aldrich	>99 %
2,6-naphthalenedicarboxylic acid	Aldrich	95 %
1,4-naphthalenediol	Eastman	
2,3-naphthalenediol	Eastman	
2-naphthalene ethanol	Aldrich	98 %
1,2-naphthoquinone	Aldrich	97 %
2-naphthoic acid	Sigma	
naphthol	Fisher	
n-pentane (non-UV)	Caledon, Georgetown, ON	pesticide grade
phenanthrene	Aldrich	98 %
1,10-phenanthroline	Fisher	
1-phenylnaphthalene	Aldrich	96 %
2-phenylnaphthalene	Fluka, Ronkonkoma, NY	
4-phenyltoluene	Aldrich	98 %
o-phthalic acid	Fisher	
protocatechuic acid	Aldrich	
pyrene	Aldrich	99 %
quinoline	Aldrich	96 %
salicylaldehyde	Sigma	
salicylic acid	Aldrich	>99 %
squalane	Sigma	
succinic acid	Fisher	
o-terphenyl	Aldrich	99 %
m-toluate	Pfaltz + Bauer	
triphenylene	Aldrich	98 %
n-undecane	Aldrich	99 %
m-xylene	Aldrich	99 %

General chemicals and reagents

ascorbic acid	Fisher	
acetone	various	
basic fuschin	unknown	
boric acid	MC+B	
bromophenol blue	Fisher	
chloroform	BDH, Toronto, ON	
crystal violet	Fisher	
dichloromethane	various	HPLC grade
N,N-dimethylformamide	Anachemia, Montreal, PQ	
ethanol	various	
ether	various	
ethidium bromide	Sigma	
glacial acetic acid	BDH	99.7 %
glucose	BDH	99.8 %
glycerol	Caledon	99.5 %
8-hydroxyquinoline	Sigma	
isopropanol	Anachemia	>98.5 %
methanol	Caledon	
methylene blue	unknown	
orange G	unknown	
phenol	Anachemia	>99%
SDS	UltraPure, Life Technol., Inc., Gaithersburg, MD	>99.5 %
silica gel (lot# 07201MQ)	Aldrich	>99 %
sodium acetate	Caledon	99 %
sodium citrate	BDH	99 %
sodium perchlorate	Aldrich	99 %
TMPD	Sigma	
Tris	ICN Biochemicals, Aurora, OH	
Triton X-100	Sigma	
Tween® 80	JT Baker, Phillipsburg, NJ	
xylene cyanol	unknown	
$CaCl_2 \cdot 2H_2O$	Anachemia	
$CuSO_4 \cdot 5H_2O$	JT Baker	
$FeCl_3 \cdot 6H_2O$	Anachemia	
$FeSO_4 \cdot 7H_2O$	Mallinckrodt, Montreal, PQ	
HCl	BDH	
$HgCl_2$	Fisher	
H_2O_2	BDH	
H_2SO_4	BDH	95 – 98 %
KH_2PO_4	BDH	99 %
K_2HPO_4	BDH	98 %
KNO_3	Anachemia	>99 %
KOH	BDH	>85 %
$MgSO_4 \cdot 7H_2O$	BDH	>98 %
$MnSO_4 \cdot H_2O$	Fisher	

NaCl	BDH	99 %
$Na_2EDTA{\cdot}2H_2O$	BDH	98 %
NaH_2PO_4	BDH	98 %
Na_2HPO_4	BDH	99 %
NaOH	BDH	97 %
Na_2SO_4 (anhydrous)	Anachemia	99 %
NH_4Cl	BDH	99.5 %
$(NH_4)_6Mo_7O_{24}{\cdot}H_2O$	Fisher	
NH_4NO_3	Fisher	
$(NH_4)_2SO_4$	Mallinckrodt	

Enzymes, antibiotics

Lysozyme	Boehringer Mannheim, Canada
Pronase	Calbiochem, San Diego, CA
Proteinase K	Boehringer Mannheim
RNase A	Sigma
Kanamycin A	Sigma
Rifampicin	Sigma

E.2. GC standards

1. The standard used for extraction and GC analysis (section 3.5.1) consisted of:

o-terphenyl	0.077 g
squalane	0.225 g

These were weighed into a 100-mL volumetric flask and brought to volume (100 mL) with DCM. The standard was stored at –20°C, sealed with Teflon tape. The standard was allowed to warm to room temperature prior to use.

1 mL of this standard was added to each culture following acidification during the extraction procedure (section 2.8.1).

2. The standard used for extraction and GC analysis (sections 3.5.2 to 3.5.4) consisted of:

o-terphenyl	0.059 g
squalane	0.243 g

These were weighed into a 100-mL volumetric flask and brought to volume (100 mL) with DCM. The standard was stored at –20°C, sealed with Teflon tape. The standard was allowed to warm to room temperature prior to use.

1 mL (section 3.5.2) or 0.5 mL (sections 3.5.3 and 3.5.4) of this standard was added to each culture following acidification during the extraction procedure (section 2.8.1).

APPENDIX F. PROTEIN DETERMINATION METHOD

Bicinchoninic acid (BCA) protein assay kit (Pierce Chemical Co., IL)

The working reagent for the BCA protein assay is prepared as a mixture of 50 parts reagent A to 1 part reagent B. Two millitres of this reagent is added to 0.1 mL of sample, blank or standard.

Standards are prepared from a bovine serum albumin (BSA) stock supplied with the assay kit (2 mg/mL provided in a 1 mL ampoule).

To prepare a set of standards, the BSA stock is serially diluted in 0.1M NaOH as follows:

Solution	volume (µL)	volume 0.1M NaOH (µL)	final BSA (µg/mL)
a	700 stock	4900	250
b	2800 a	2800	125
c	2100 b	3150	50
d	2800 c	2800	25
e	700 d	2800	5

These standard stocks were then divided into approximately 1-mL aliquots in sterile microfuge tubes and stored at 4°C. This allowed multiple series of assays to be conducted with the same set of standards. Standards must be included with each assay incubation as the assay has no true endpoint (colour continues to develop post-incubation) and thus, readings may vary slightly from assay to assay for samples containing the same protein concentration.

The blank incubated in the assay is 0.1 mL 0.1M NaOH (the diluent for all standards and the solution used to lyse the cell samples)